PCT/EP 00/01903

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REC'D 28 AUG 2000

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Patentanmeldung Nr. **Patent application No.** **Demande de brevet n°**

99104519.6

PRIORITY DOCUMENT

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 99104519.6
Demande n°:

Anmeldetag:
Date of filing: 06/03/99
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
ARTEMIS Pharmaceuticals GmbH
51063 Köln
GERMANY

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
Stable recombinant influenza viruses free of helper viruses

In Anspruch genommene Prioriät(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/86, C12N7/01, C12N5/10, A61K39/00, A61K39/145, A61K48/00

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

Stable Recombinant Influenza Viruses Free of Helper Viruses

The invention relates to a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus; a method for the production of said recombinant influenza virus, a method for constructing specific ribozyme-sensitive influenza carrier strains; pharmaceutical compositions comprising said recombinant influenza viruses; and the use of said recombinant influenza virus for preparing medicaments for vaccination purposes.

Redesigning influenza virus into a vector system for expression of foreign genes similar to what has been achieved in several other thoroughly studied viruses such as adenovirus, retrovirus, Semliki Forest virus or Rabies virus has the advantage of an industrially well established mode of cheap propagation for influenza in fertilized chicken eggs leading to rather high titers (above 10^{10}/ml). On the other hand none of its genes may be deleted from the influenza genome according to our present knowledge, and give room for large-size foreign insertions. Only small fragments of foreign polypeptide chains such as B cell epitopes (10 to 15 amino acids) may be inserted into selected positions within two of the viral proteins, i.e. in exchange for one of the variable antigenic regions located at the surface of hemagglutinin (Muster et al., Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. J. Virol. 69 (11), 6678-6686 (1995)), or into the stalk sequence of viral neuraminidase (Garcia-Sastre and Palese, The cytoplasmic tail of the neuraminidase protein of influenza A virus does not play an important role in the packaging of this protein into viral envelopes. Virus Res. 37, 37-47 (1995)), and be stably maintained as functional fusion proteins. Constructs of this kind turned out to be useful for experimental vaccination in a few cases studied, but only rather few clearly defined epitope sequences (of ten to twelve amino acids each) are known today, and some of them might also be misfolded within such restricted fusion protein positions, or in other cases interfere with the correct tertiary structure and function of their host polypeptide chains.

Incorporation of a full-size foreign protein into influenza via reverse genetics, encoded by an independent ninth vRNA molecule in addition to its regular set of eight standard vRNA segments is without special provisions only transiently possible (Luytjes et al., Amplification, expression, and packaging of a foreign gene by influenza virus. Cell 59, 1107-1113 (1989);

Enami et al., An influenza virus containing nine different RNA segments. Virology 185, 291-298 (1991)). In the absence of a continuous selective pressure any additional recombinant vRNA segment cannot be stably maintained as long as a wildtype promoter sequence is used on that ninth vRNA segment, and it will inadvertently be lost after few steps of propagation. Using a different system of influenza reverse genetics developed in our laboratory (Zobel et al., RNA polymerase I catalysed transcription of insert viral cDNA. Nucleic Acids Res. 21, 3607-3614 (1993); Neumann et al., RNA polymerase I-mediated expression of influenza viral RNA molecules. Virology 202, 477-479 (1994)), which was built around *in vivo* synthesis of recombinant vRNA molecules by cellular RNA polymerase I transcription of the respective template cDNA constructs, promoter-up mutations have been designed by nucleotide substitutions (Neumann and Hobom, Mutational analysis of influenza virus promoter elements *in vivo*. J. Gen. Virol. 76, 1709-1717 (1995)). When these are attached to a recombinant ninth vRNA segment its increased transcription and amplification rate will not only compensate for the losses suffered spontaneously, but even cause accumulation of the foreign vRNA segment during simple viral passaging, in the absence of any selection. However, due to its over-replication relative to all of the regular influenza vRNA segments (which of course are connected to wild-type promoter sequences) after catching up with the others the foreign segment will become over-abundant. This increasingly will result in viral particles that have incorporated several copies of recombinant vRNA, but no longer have a full set of all eight standard segments incorporated among an average of about 15 vRNA molecules present within a virion. Such particles are defective and will not result in plaque formation, hence after an initial increase of recombinant viral particles during the first steps of propagation a dramatic decrease is observed, usually at the third or fourth step of viral passaging, depending on the size of the recombinant vRNA and the level of the promoter-up mutation attached. A balanced situation with regard to the insert length and the level of promoter activity can be achieved, and has been propagated in a particular case over 11 passages, with essentially stable levels of recombinant viruses among a majority of helper viruses (around 80%) during these steps. If a full-level promoter-up mutation is used (1104 or the new variant 1920, see below) a balanced-level propagation is reached in conjunction with a recombinant vRNA size of 4000 nucleotides (unpublished).

In all of these preparations, both in transiently achieved increased yields (up to 40% of recombinants after three or four steps of viral passage), and in a balanced propagation of

recombinant influenza viruses (10 - 20%) the respective viral progeny inadvertantly constitute mixtures with a majority of non-recombinant helper viruses. These result both from a statistical mode of packaging vRNA molecules into a virion (the ninth segment may not be co-packaged), and from the fraction of cells solely infected by helper virus.

The problems of fractional yields and of instability during viral propagation of recombinant influenza are the problems to be solved with the present invention.

Starting out from two observations in this laboratory which are discussed below and which concern two hitherto unsuspected properties of influenza viral RNA polymerase in its interaction with terminally adapted influenza-specific RNA molecules, a new technique for the construction of stable recombinant influenza viruses was found.

As previously described in WO 96/10641 plasmid constructs are designed to generate influenza vRNA-like molecules *in vivo* by cellular RNA polymerase I transcription following plasmid DNA transfection into tissue culture cells, and to this end contain flanking rDNA promoter and terminator elements, externally located relative to any cDNA constructs in between. The resulting recombinant vRNA molecules are designed to contain 5' and 3' recognition sequences for influenza viral RNA polymerase, which however carry up to five nucleotide substitutions (in promoter-up mutant pHL1920) resulting in a substantial increase of expression over wildtype influenza promoter levels. While recombinant pseudoviral RNA is initially transcribed by RNA polymerase I, further amplification and mRNA transcription depends on the presence of viral RNA polymerase and viral nucleoprotein in the cell, which generally are provided by infection of a helper virus. As a consequence the progeny viral yield will constitute a mixture of recombinant viruses together with a majority of wild-type helper viruses.

In the new technique the recombinant vRNA-like molecules as transcribed by RNA polymerase I are constructed as ambisense RNA double segments, with one reading frame (an influenza gene) in sense and a second one (a foreign gene) in anti-sense orientation, or vice versa. In such constructs both reading frames will be expressed via the cap-snatching mode, even if at different levels. Again, infection by helper virus is required to provide the necessary viral early and late proteins for genetic expression and virion packaging. However, the particular helper virus used in the new method is a recombinant virus carrying 2x2 specifically designed ribozyme targets inserted into the flanking non-coding sequences of one

of its eight vRNA segments. The viral segment chosen to become ribozyme-sensitive is always identical to the one used in constructing the recombinant ambisense RNA molecule, as the viral carrier gene in covalent linkage with an additional foreign gene.

Recombinant influenza viruses produced in this way through RNA polymerase I transcription of an ambisense viral RNA molecule followed by infection with that specifically designed type of ribozyme-sensitive helper virus will carry one of the influenza genes twice, once within that ribozyme-sensitive helper vRNA segment, and a second time within the ribozyme-resistant ambisense segment. Recombinant viruses of this type are again obtained initially only as a mixture together with a majority of non-recombinant helper viruses. A progeny viral passage through tissue culture cells (293T) which before have been transiently transfected with plasmid constructs expressing the respective double-headed ribozyme will (in one step) inactivate the ribozyme-sensitive segment by a factor of up to 100. One or two rounds of such ribozyme treatment *in vivo* will at the same time (a) purify the recombinant virus from its non-recombinant helper contaminants, and (b) delete the sensitive vRNA helper segment from within the initial (additive) recombinant virus.

As a result recombinant influenza viruses are isolated along this several-step procedure, which are free of contaminating helper viruses and carry seven regular and one ambisense vRNA segments, all in a balanced replication mode. Their recombinant nature is stably maintained because of a covalent junction between one of the viral genes and the full-size foreign gene inserted, a situation achieved here for the first time, via constructing an influenza ambisense RNA segment. The whole procedure is independent of any (selectable) phenotype, and can be applied to either of the eight influenza vRNA segments. After establishing a first ambisense vRNA segment carrying a single foreign gene it can also be repeated all over for inserting a second foreign gene within another ambisense RNA segment of the same constitution in principle.

Stable recombinant viruses of the type described can be used for cheap propagation in fertilized eggs, either for production of those recombinant viruses themselves or for production of foreign proteins or glycoproteins encoded by them, and hence find application in (glyco)protein production or in providing vector systems for somatic gene therapy or in being used as vaccination agents.

Thus, the present invention provides

(1) a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus, wherein

(a) at least one of the regular viral RNA segments has been exchanged for a vRNA encoding a foreign gene; and/or

(b) at least one of the regular viral RNA segments is an ambisense RNA molecule, containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement;

(2) a method for the production of recombinant influenza viruses as defined in (1) above comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo,* in antisense, sense or ambisense design,

(b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA segments, and

(c) thereafter selective vRNA inactivation through ribozyme cleavage;

(3) a method of constructing influenza carrier strains carrying one or more ribozyme target sites (of type one) in vRNA flanking positions comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, carrying two different 3' promoter sequences in tandem, which are separated by a second type of ribozyme target sequence, and which carry the said internal ribozyme target sites of type one;

(b) followed by infection of an influenza wildtype strain;

(c) thereafter amplification through simple steps of viral propagation; and

(d) finally isolation through removal of their external 3' promoter sequence by ribozyme cleavage through infection of cells expressing ribozyme type 2, followed by plaque purification;

said method being suitable for the constuction of an influenza carrier strain required for step (b) of (2) above;

(4) a ribozyme-sensitive influenza carrier (helper) strain obtainable by the method of (3) above;

(5) a pharmaceutical composition comprising a recombinant virus as defined in (1) above;

(6) use of a recombinant virus as defined in (1) above for preparing a medicament for vaccination purposes;

(7) use of a recombinant virus as defined in (1) above as vector systems in somatic gene therapy, for transfer and expression of foreign genes into cells (abortively) infected by such viruses, either in *ex vivo* or *in vivo* application schemes; and

(8) a method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus as defined in (1) above as expression vector.

Moreover, the invention provides a method for preventing/treating influenza utilizing a recombinant virus as defined in (1) above, i.e., a vaccination method utilizing said recombinant virus.

According to the present invention "influenza virus" embraces influenza A virus, influenza B virus and influenza C virus. A preferred recombinant virus of the invention is where one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged into foreign glycoprotein(s) or into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

Further preferred recombinant viruses of the invention are where the terminal viral RNA sequences, which are active as the promoter signal, have been varied by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence.

Further preferred embodiments of the invention are set forth herein below.

A. Construction of influenza helper virus strains carrying ribozyme target sequences in flanking positions within either of the vRNA segments

A.1. Influenza RNA polymerase will initiate transcription and replication from promoter structures located at internal positions in an RNA molecule, not only from the natural position at both ends of a vRNA molecule:

This is true in particular for promoter-up variants in RNA-internal location due to their enhanced binding affinity for viral polymerase. Not only 3' end extensions are tolerated in RNA-internal promoter recognition (Fig. 1), but also 5' extensions as well as extensions at both ends of the RNA template molecule, containing noncomplementary as well as complementary sequence, i.e. potentially present as a double-stranded extension. Finally, also an extension by way of duplication of the promoter sequence (active or inactivated) leads to mRNA transcription and CAT expression, initiated from the active pair of 5' and 3' promoter halves, irrespective if in external or in internal or even in an oblique localization (active 5' promoter sequence in external, active 3' promoter sequence in internal position, or vice versa). RACE-determination of the resulting 5' and 3' ends of viral mRNA and cRNA, i.e. the products of transcription and replication reactions for several of the extended template vRNA constructs proves an exact recognition and sequence-specific initiation at a position equivalent to regular 3' position $\bar{1}$: all of the various template extensions are lost in every product RNA molecule, most likely after only one round of replication.

A.2. Bicistronic (tandem) vRNA molecules carrying an additional 3' specific promoter sequence in a central position between its two genes can be used for an indirect selection method for recombinant influenza viruses:

The method described is applicable for any foreign gene (e.g. CAT) without a selection potential of its own, if inserted into the distal mRNA position (proximal vRNA position, in anti-sense orientation) behind a carrier gene (e.g. GFP) in the proximal mRNA position, able to serve as a transient selection marker. The carrier gene which is used for selection will get lost spontaneously during further propagation. These constructs are equivalent to a 3' extension of the template vRNA by a full-size gene of 750 nucleotides up to a second 3' promoter sequence, in terminal location. While in the set of experiments shown in Fig. 2 the external 3' promoter sequence is maintained throughout as the same promoter-up variant (1104), the internal 3' promoter sequence has been varied to include a full-level promoter-up variant (pHL2270, containing promoter mutant 1104), a medium-level promoter variant (pHL2350, promoter mutant 1948), a wildtype promoter construct (pHL2629), and a construct carrying an unrelated central sequence in an otherwise identical design (pHL2300).

Due to the presence of two 3' promoter sequences in conjunction with a single 5' promoter sequence an alternating interaction between them will constitute one or the other active

promoter structure (see Fig. 3). While the external location with an adjacent RNA 3' end may have a structural advantage, this appears to be compensated by the shorter distance in an interaction between the 5' sequence and the central 3' sequence in constituting the internal promoter, such that the competition between the two primarily reflects the various internal 3' promoter allele sequences used, compare Fig. 2B and activity ratios indicated above and below the lanes. Translation of the mRNA-distal gene (CAT) is only observed following an internal initiation at the bicistronic vRNA template, resulting in a spontaneous deletion of the mRNA-proximal (vRNA-distal) gene, GFP, compare right half of Fig. 3. In complementary analyses GFP fluorescence is observed initially for all of the bicistronic constructs, but gets lost on a faster rate from pHL2270 transfected and influenza infected cells (not shown), and will stay unchanged in pHL2300-treated cells. The indirect selection system based on bicistronic (tandem) molecules as designed here and demonstrated for reporter genes GFP and CAT can be used for any other gene without distinct phenotype upon insertion behind an unrelated carrier gene with properties useful in selection. – In employing that technique an initial phase of (repeated) positive selection for infected cells expressing that proximal trait (e.g. by FACS or by magneto-beads) will be followed by a second phase with negative selection, i.e. against that fraction of infected cells still exposing the same property.

A.3. Isolation of an influenza strain designed to carry 2x2 flanking ribozyme target sequences at the 5' and 3' end of vRNA segment 4 coding for hemagglutinin:

The above scheme for an indirect selection of any foreign recombinant gene behind a proximal carrier gene is further modified by deleting the carrier gene altogether. Instead, both 3' terminal promoter sequences (mutant and wildtype) follow each other at a short distance, separated only by a specific, repetitive ribozyme target sequence, - different from other target sequences to be described further below. The cDNA insert following after the second 3' promoter sequence consists mainly of a regular hemagglutinin (H7) coding sequence, however both the 5' and 3' vRNA terminal regions of the insert carry that other ribozyme target sequence (different from the first target sequence mentioned above) inserted in either location in tandem duplication (pHL2969, see Fig. 14).

Due to a superior replication supported by the promoter-up variant located in 3' external position the recombinant HA segment attached to that promoter sequence will become enriched during the first steps of viral propagation, while the originally dominating HA

segment of the helper virus which is under control of a wildtype promoter sequence is consecutively reduced and finally is no longer detectable among viral progeny. This result is documented by RT-PCR analysis of consecutive viral populations as obtained in that stepwise propagation procedure, see Fig. 4.

In the next step the viral lysate is twice passaged via infection of cell culture cells (293T) that before have been DNA-transfected by plasmid pAM403 (Fig. 15). This construct has been specifically designed to express a hammerhead ribozyme with flanking sequences complementary to the repetitive GUC-containing target sequence, as present twice in between the external and internal 3' promoter signals in the recombinant HA vRNA segment, see Fig. 5. In this way the extra promoter sequence is cut off from the finally resulting recombinant HA vRNA. Its promoter-up activity was useful in achieving an initial increase in the concentration of recombinant HA vRNA over wildtype HA vRNA, and in finally excluding the latter from further propagation. However, for the same reason that high activity of the promoter variant will cause instability in the resulting viral progeny, and an effective 'substitution' at this time through ribozyme cleavage by the internally located promoter signal, wild-type or slightly improved, will restore stability to the progeny viruses, with all of their eight vRNA segments now brought back in balance to each other. Due to the ribozyme cleavage site at 26 nucleotides 3' of the wild-type promoter sequence (see Fig. 5), in the initial stage that promoter signal is situated in a vRNA-internal location, extended by a 3' terminal sequence of 26 nucleotides. According to the data presented in Fig. 1 this should cause a transient slight reduction in activity, resulting however in one step in regular viral mRNAs and cRNAs, with any initially remaining extra sequence being lost from the finally resulting recombinant HA vRNA.

Progeny viruses still carrying an external promoter-up sequence (before ribozyme treatment or due to incomplete reaction) will not cause any plaque, due to over-replication of one vRNA segment relative to all others which results in a high load of defective particles. However, progeny viruses which have lost that external promoter element due to ribozyme cleavage will yield regular plaques due to a balanced mode of replication for all eight wild-type or recombinant vRNA segments. Hence plaque purification is used for isolating a pure influenza viral strain carrying 2x2 ribozyme targets in its recombinant HA vRNA segment, with its termini reduced to the wild-type promoter sequence. The nature of the viral strain isolated has been confirmed in this regard by RT-PCR analysis, see Fig. 4.

The above isolation procedure resulting in influenza viral strains carrying 2x2 flanking ribozyme target sequences has been performed twice for the HA coding segment (segment 4) to obtain two different isolates with regard to the orientation of the ribozyme target sequences. In one of the isolates (vHM41, see SEQ. ID NO: 3) the tandem target sites have been inserted into the HA vRNA non-translated sequence both in 5' and 3' location, while according to the second design that 5' tandem target sequence has been included in an inverted orientation, such that it is now present in the cRNA 3' sequence instead (vHM42).

In another experiment the same procedure was used to isolate an influenza strain carrying 2x2 tandem target sites within the 5' and 3' flanking positions of segment 8 vRNA, i.e. coding for genes NS1 and NS2 (vHM81; see SEQ.ID NO:4). And in principle the same could be done for any other influenza segment, in particular since only the reading frame cDNA sequence has been exchanged from HA to NS, with all of the flanking elements directly responsible for that procedure remaining in place, unchanged.

A.4. Ribozyme cleavage and vRNA segment exchange using ribozyme-sensitive influenza strains as helper viruses:

In an initial model experiment a range of ribozyme type and target site locations was probed in designing a series of CAT reporter gene vRNA constructs (analysed in the presence of a surplus of wildtype helper virus) in 293T cells. While all of the ribozyme constructs adhered to the hammerhead model, with 10 to 12 nucleotides of complementary sequence flanking on either side of the GUC target site, these ribozyme constructs varied from monomer to dimer to trimer repetitions. Ribozyme containing mRNAs were synthesized *in vivo* via the basic vector plasmid pSV2-*neo*, i.e. using the efficient p_{SVe} RNA polymerase II promoter element for expression, and the SV40 origin signal for plasmid amplification, in a cell line (293T or cos-1) with an incorporated SV40 T antigen gene. In addition the pSV2-*neo* mRNA includes the small, 63 nucleotide intron sequence of the SV40 early mRNA which is supposed to be spliced very slowly, thereby extending the pre-mRNA half-live in the nucleus. Each of the pSV2-*neo*-ribozyme plasmid constructs was transfected into 293T cells. Thereafter, recombinant viruses containing dimer target sites either near one end of the molecule only, or near both ends have been used for infection of the transfected cells. Relative activities of ribozyme constructs versus vRNA target sites have been measured via inactivation of CAT acetyl transfer rates in the cell lysates obtained at 8 h post infection (Fig. 6). The highest

activities were observed for dimer ribozymes acting on vRNA molecules carrying 2x2 target sequences on both ends of the molecule, either in vRNA 5' and 3' location, or in vRNA 3' and cRNA 3' location, i.e. with an inversion of the target site sequence at the vRNA 5' end. Consequently, the two constructs carrying tandem ribozyme double targets within both of their non-translated vRNA flanking sequences have been used in the design of ribozyme-sensitive influenza virus strains as described above, with both variants isolated for segment 4 (HA), and one of them for segment 8 (NS). In complementary correspondence the hammerhead ribozyme plasmid used has also been constructed as a double headed structure with flanking sequences as shown in Fig. 7 (pAM424; for its detailed structure, see Fig. 16).

The three target site-containing influenza strains isolated as described above have been analysed for their sensitivity against ribozyme cleavage by infection of 293T cells, which had been DNA-transfected 18 h earlier by ribozyme-producing plasmid pAM424, at DNA-transfection rates between 60 and 70 % (as observed in parallel transfections using GFP-expressing plasmid pAM505). Inactivation rates in these one-step control experiments were between 90% and 99% for all three ribozyme-sensitive strains, in their extent mainly depending upon the actual DNA-transfection rates achieved in individual experiments.
In the next step both isolates of HA-coding ribozyme-sensitive viruses, vHM41 and vHM42, have been used in marker-rescue experiments. Here, 293T cells have been first DNA transfected by HA-variant cDNA construct pHL2507 (see Fig. 17), followed after 18 h by vHM41 or vHM42 virus infections at moi 1. The resulting viral supernatant containing e.g. a mixture of ribozyme-sensitive vHM41 carrier virus and pHL2507/vHM41 recombinant virus is propagated in an intermediate step on MDCK cells, which also results in an increase in the fraction of recombinant viruses. Thereafter the resulting virus-containing supernatant is passaged through 293T (or cos-1) cells, which in advance have been transiently transfected by ribozyme-producing pAM424. As may be concluded from the above experiment (Fig. 6) and shown in Fig. 8 vHM41- or vHM42-derived ribozyme-sensitive HA vRNA segments are expected to be inactivated by pAM424-produced ribozyme down to a remaining level of about 1% to 10% (mainly present within cells that are infected, but not DNA-transfected).

Instead, the substitute HA vRNA which originated from pHL2507 plasmid DNA (ribozyme-resistant) becomes exclusively incorporated into progeny virions. For further purification and

viral propagation these have been passaged a second time through 293T cells, again in advance DNA-transfected by pAM424, and after another amplification step on MDCK cells the resulting viral preparations have been used for RT-PCR analysis. The resulting viral populations in these marker rescue experiments only contain HA-vRNA molecules derived from pHL2507, which in their PCR analyses are of intermediate size relative to wildtype HA-vRNA, and vHM41- or vHM42-derived HA-vRNAs, respectively.

Consequently, a set of ribozyme-sensitive influenza strains with targets inserted individually into every vRNA molecule may be used for such one-step marker rescue experiments in general, i.e. for vRNA segment exchange reactions performed in a directional way for any of the eight influenza vRNA segments, without requirement for a selectable change in phenotype (genetic marker).

B. Expression of two gene products from ambisense bicistronic influenza vRNA

B.1. The influenza cRNA promoter is active in antisense viral mRNA transcription according to the cap-snatching mode of initiation:

While the vRNA template of influenza virus is known to be active in viral mRNA as well as cRNA synthesis, the cRNA template has been described so far only to produce vRNA molecules, as a second step in viral replication. The potential activity of the cRNA promoter in initiating also viral mRNA transcription has not been analysed or even suspected so far, since no antisense (vRNA) reading frame can be detected in any of the viral RNA segments. Also, no U_5/U_6 template sequence element is present in any of the viral cRNAs in an adjacent position to its 5' promoter structure as is the case for all viral vRNAs. This element is known to serve as a template sequence for mRNA terminal poly-adenylation, in repetitive interaction. However, when both elements are provided through reconstruction of an artificial influenza cRNA segment: a reading frame in opposite orientation (CAT), and a U_6 template element in 5' adjacent location, CAT expression can indeed be observed, see pHL2583 (see Fig. 18) in Fig. 9. Similar to the vRNA promoter the cRNA promoter activity is improved by (the same) promoter-up mutations, which essentially consist of basepair exchanges according to the 'corkscrew' model. This model apparently also holds for the cRNA promoter structure as analysed in a stepwise manner in Fig. 9. While the cRNA promoter has to be superior over the vRNA promoter in its initiation of replication, since the vRNA/cRNA product ratio was

determined to be around 10:1 (Yamanaka et al., *In vivo* analysis of the promoter structure of the influenza virus RNA genome using a transfection system with an engineered RNA. Proc. Natl. Acad. Sci. USA 88, 5369-5373 (1991)), the cRNA promoter is observed to be inferior to the vRNA promoter in its initiation rate of transcription (compare pHL2583 with pHL1844 in Fig. 9), at least for all promoter variants tested so far.

A RACE analysis for determination of the 5' ends in pHL2583 cRNA promoter transcribed mRNAs proved this initiation to occur according to the cap-snatching mode, in complete equivalence to standard vRNA promoter controlled transcription initiation.

B.2. Development of ambisense influenza constructs for consecutive expression of two genes (GFP and CAT) from a single viral RNA:

For a bidirectional transcription and translation of influenza RNA segments the two reporter genes GFP and CAT have been arranged in opposite orientation to each other, and the flanking 5' and 3' promoter sequences (adhering to promoter-up variant 1104) had to be reconstructed to include a U_6 poly-adenylation element in either orientation in a 5' promoter adjacent position. This requirement necessarily resulted in a promoter-adjacent 5'-U_6/3'-A_6 complementary structure, both in the vRNA and cRNA terminal sequence (see Fig. 10), which had to be tested for its promoter activity, in either orientation. Therefore, the convergent pair of reporter genes GFP and CAT has been inserted in both orientations, such that CAT transcription is initiated by the vRNA promoter in one construct (pHL2960), and by the cRNA promoter in the other (pHL2989, Fig. 19), and vice versa for GFP expression from both ambisense constructs. In addition, also the CAT gene only has been inserted in either orientation between the 5' and 3' elements of that ambisense promoter, with CAT transcribed by the vRNA promoter in one case (pHL2959), and by the cRNA promoter in the other (pHL2957). The whole set of constructs allows for a direct comparison with corresponding reference constructs carrying a regular vRNA promoter (pHL1844) or cRNA promoter structure (pHL2583), i.e. carrying only the 5'-adjacent U_6 sequence element and no 3'-A_6 counterpart. The two groups of constructs also differ in insert size, since a single inserted gene roughly accounts for 750 nucleotides, the convergent set of two genes for 1500 nucleotides, with the distal half of both mRNAs in this case remaining untranslated, a situation unusual for influenza viral mRNAs.

As is demonstrated in Fig. 10B for CAT expression of the various ambisense constructs all of them are able to initiate transcription in both orientations, even if at different levels with regard to their vRNA and cRNA promoter-dependent expressions, and also with regard to the insert lenghts and convergent arrangements of the GFP/CAT versus CAT-only constructs. Analysis of the GFP expression rates (not shown) yields complementary results, i.e. again vRNA promoter-controlled GFP expression is superior over cRNA promoter expression of GFP. Therefore, individual ambisense clones either show an asymmetric high expression of ~~GFP and low expression of CAT (pHL2960) or vice versa (pHL2989), depending on their~~ orientation of reading frames with regard to the external vRNA and cRNA promoter. Fig. 10C also demonstrates successful propagation of recombinant viruses containing ambisense RNA molecules, which proves survival through amplification, packaging into virions, and expression of both mRNAs in infected MDCK cells (including besides CAT also GFP expression).

B.3. Construction of a superior promoter-up mutation, pHL1920, to be used for improved rates of cRNA promoter expression in ambisense constructs:

An extended analysis of promoter variants, in particular of complementary double exchanges according to the 'corkscrew' model yielded among others variant pHL1920 (Fig. 20) with CAT activity rates considerably above (125-130% of) the rates observed for standard promoter-up variant '1104' (as present in pHL1844). The '1920' promoter-up variant consists of altogether 5 nucleotide substitutions relative to the wildtype promoter sequence, both in the 5' promoter element (2), and the 3' promoter element (3). The structure of this variant and the whole set of complementary double exchanges is presented in Fig. 11, together with the respective CAT activity measurements, in vRNA promoter constructs. vRNA promoter-up variants also show similarly improved expression in (ambisense) cRNA constructs, even if at generally lower levels than in vRNA constructs. cRNA promoter-up expression is observed at levels similar or somewhat (2x-5x) above the *wild-type* vRNA promoter rate, while vRNA promoter-up constructs show CAT expression rates increased up to 20 or 25 times the wild-type vRNA promoter level. In either case expression rates also depend on the size of the insert, with promoter activity rates decreasing with increasing lengths of the influenza RNA molecules to be transcribed.

B.4. Influenza recombinant viruses containing a foreign gene (CAT) in covalent ambisense linkage with one of the viral genes (HA, NS1/NS2):

The principle solution in designing stable recombinant viruses based on the new properties observed for influenza transcription and replication signals consists in constructing viruses which contain a foreign gene in covalent linkage with one of the (indispensable) viral genes, in ambisense bicistronic organization. Preferably the viral gene is connected to the cRNA promoter, while vRNA promoter expression is used for expression of the foreign gene at rates considerably above the viral mRNA synthesis. The promoter-up variant chosen for constructing the ambisense RNA segment intends to bring its cRNA promoter expression (approximately) into balance with all other viral gene expression levels, which are controlled by wild-type vRNA promoters located at the termini of the seven ordinary influenza segments; the respective choice has to take into consideration the overall length of the ambisense segment.

Isolation of the ambisense recombinant virus employs an RNA polymerase I-transcribed ambisense cDNA construct, which will give rise *in vivo* to ambisense cRNA-type molecules, see Fig. 12. The plasmid DNA transfection mixture used in this step with 293T cells in addition may or may not contain four 'booster' plasmids which under p_{CMV}-control produce the four early influenza proteins from non-viral mRNAs: NP, plus PB1, PB2, and PA, i.e. the three subunits of viral polymerase (Pleschka et al., A plasmid-based reverse genetics system for influenza A virus J. Virol. 70, 4188-4192 (1996)), which will increase in a pre-amplification step the copy number of that ambisense viral cRNA segment. At 18 h post transfection the 293T cells are infected by a ribozyme-sensitive influenza strain, e.g. vHM41, which will supply (again) early and also late viral RNAs. The resulting supernatant which contains a mixture of vHM41 carrier virus and vHM41-derived ambisense recombinant virus (nine vRNA segments) is then passaged directly or via an intermediate step of amplification on MDCK cells onto 293T cells that have in advance been DNA-transfected by ribozyme-producing pAM424. Here, the ribozyme-sensitive vRNA segment of vHM41 will be cleaved at its 2x2 target sites by pAM424 specific ribozymes. In recombinant viruses the vRNA gene lost in this way is re-supplemented through its presence within the ambisense segment. The virus-containing supernatant is passaged for amplification and further purification through ribozyme treatment a second time on 293T cells which again have been pretreated by pAM424 DNA transfection.

Absence of ribozyme-sensitive vRNA, and presence only of ambisense RNA in RT-PCR analysis at this stage allows for further amplification on MDCK cells and a final virus stock preparation on embryonated chicken eggs. CAT assays can be used to analyse for the presence and monitor the activity of this model foreign gene through the various steps of isolation and propagation as well as document technical improvements that might be worked out for one or more of the processive stages.

C. Examples for application of helper-free, stable recombinant influenza viruses

C.1. Incorporation of reporter gene GFP in NS/GFP or HA/GFP ambisense segments:
Recombinant viruses of this type will allow to follow-up on influenza infection instantly and continuously in individual infected cells, which may also be counted or documented by fluorescence photography or FACS sorting. With improved temperature resistance (Siemering et al., Mutations that suppress the thermosensitivity of green fluorescent protein. Curr. Biol. 6, 1653-1663 (1993)) GFP expression becomes visible at 2 h after infection and shows bright fluorescence after 4 h p.i., it will be possible to follow-up on the spread of viral infection by GFP fluorescence. Stable fluorescence in abortively infected cells e. g. observed in *ex vivo* treatment of dendritic cells similarly supports a follow-up on their reincorporation into animals; other genes may be incorporated by ambisense vRNA into dendritic cells in the same way.

C.2. Construction of glycoprotein CSFV-E2 carrying influenza, helper-free:
The glycoprotein E2 of CSF virus has been incorporated previously into influenza both as an HA-anchor fusion protein within the viral envelope, and as an additional, unstable ninth vRNA segment into its genome (Zhou et al.; Membrane-anchored incorporation of a foreign protein in recombinant influenza virions. Virology, 246, 83-94 (1998)). Stabilization is now achieved through an ambisense connection with either of the regular viral RNA segments (NS or HA) which also allowed to reach a level of 100% recombinant viruses instead of an hitherto only 20% (Fig. 13), since all carrier viruses are destroyed through ribozyme action, see Figs. 8 and 12. The helper virus containing preparation has already been used successsfully as a vaccine against CSFV infection (antibody titers of 1:40000); the increase achieved in recombinant viruses allows a further improvement in that regard. Also (cost-

effective) propagation in fertilized chicken eggs has become possible due to its stable incorporation of the foreign gene as a covalent ambisense construct.

C.3. Construction of hepatitis C glycoprotein-carrying recombinant influenza viruses as a candidate vaccine:

Hepatitis C virus is a close relative of CSF virus (hog cholera virus), and in particular its set of two glycoproteins, small-size E1 and larger-size E2, is closely related in structural detail and presumably also in function to the corresponding CSFV proteins. An incorporation of HCV-E2/HA fusion proteins into influenza viral envelopes has been achieved in analogy to the CSFV-E2/HA incorporation. In addition, incorporation of an anchor-fusion glycoprotein HCV-E1/HA or both together (in NS *and* HA ambisense junctions) allows further variations in constructing an influenza-based vaccine for hepatitis C. In analogy to CSFV-E2, neutralizing antibodies are expected to be directed against particular epitopes of HCV-E2, presented in essentially native conformation at the influenza viral envelope.

C.4. Stable incorporation of selected influenza T-cell epitopes in ambisense constructions:

Influenza infection is known to result in both, antibody production against that specific viral strain or indeed its epitopes that are located mainly at the surface of HA, and in an increase of specifically primed cytotoxic T-lymphocytes, stimulated by T-cell epitopes primarily located within the essentially invariable core structure of the NP protein. While the humoral response will result in life-long immunity against *that particular* strain of influenza or its epitope structures, the T-cell response will be lost or severely reduced some time afterwards, such that its specificity against influenza in general will fall below protective levels. One way in trying to increase that level of cellular immunity is to enhance the response or recruitment of influenza-specific CTL cells by increasing the level of T-cell epitopes in the infected cell and hence its presentation on the surface by MHC-I receptors. This is achieved by combining in an ambisense construct the HA gene and a series of repeated T-cell epitope sequences as present in the influenza NP gene, in a model design specific for the BALB/C mouse MHC-I allele. Here, the promoter-up expression rate is realized (in vRNA promoter-controlled initiation) for expression of the repetitive epitope polypeptide chain. Alternatively or in addition a controlled secretion of an interleukin can be achieved from recombinant influenza-infected cells, upon ambisense incorporation of the respective gene preferrably into the NS

segment. The interleukin to be chosen for this purpose (IL-12 or other) is selected to enhance the longevity of influenza-specific CTL cells or its conversion into corresponding memory cells. In this way an ambisense vaccine strain against influenza itself is achieved with expected protective capacity against influenza in general.

C.5. Exchange of influenza glycoproteins against foreign viral glycoproteins (VSV-G):

The 'marker rescue' experiment described above (section A.4.), i.e. an exchange of one HA gene (ribozyme-sensitive) for another (ribozyme-resistant) can also be used for an exchange of HA for an entirely different glycoprotein, such as the vesicular stomatitis virus G protein, as long as it is attached to the HA anchor segment. Effective incorporation depends always on that C-terminal tail sequence and its interaction with underlying matrix protein M1, and therefore, all constructions consist of fusion proteins in direct analogy to CSFV-E2. VSV-G with or without a foreign anchor sequence has been shown in several other viruses to be able to substitute for the original glycoprotein and to result in infectious viruses with VSV-G specific host-ranges (e.g. in retroviruses, rabies virus, measles virus). G protein in VSV itself as well as on the surface of foreign viruses is the only glycoprotein required for all of the consecutive steps in infection. Insertion of VSV-G instead of HA in recombinant influenza viruses leaves the second glycoprotein, neuraminidase, without any function, which then will get lost spontaneously from the recombinant viruses. This will further increase the capacity for an addition of foreign genes, beyond the gain resulting from an exchange of the larger HA for the smaller VSV-G, which might be used for an addition of ambisense constructs.

The invention is further illustrated in the accompanying figures.

Description of the figures

<u>Fig.1. 3' nucleotide extensions of influenza vRNA template molecules:</u>

(A) Murine B82 cells have been transfected by plasmid cDNA constructs designed to be transcribed into influenza vRNA molecules by RNA polymerase I *in vivo*, followed after 20 h by standard $FPV_{Bratislava}$ helper virus infection, at an moi of 1 to 3. In addition to reference plasmid pHL2024 (no extension), related cDNA constructs carrying extensions of 1 to 50 bp, and hence extended by 1 to 50 nucleotides at the resulting vRNA 3' ends were used in parallel

transfections; template extensions are marked at the top of the figure. Cell lysates prepared at 8 h post helper virus infections were used for CAT reactions using in one round 50 µl of cell lysate each, and in further analyses 5µl and 0.5 µl of lysate (not shown). Relative yields were determined in comparison to reference plasmid pHL2024, as indicated below the figure, with calculations restricted to those CAT assays showing less than 40% of substrate consumption, in three or more independent experiments.

(B) Viral passage of B82 supernatants containing recombinant influenza virus onto MDCK cells, at an moi of 2 to 4, in average. Again at 8 h post infection cell lysates have been prepared and used for CAT assays. Relative yields as indicated below the figure have been determined in comparison to pHL2024 the same way as in (A), using 0.5 µl of cell lysate in each case. (The 50 µl CAT assays as shown here and also in the following figures intend to give an immediate impression of relative activities at always the same level, while the actual measurement data as indicated below the lanes are obtained at various appropriate enzyme concentrations relative to reference pHL2024.)

Fig. 2. Propagation of recombinant influenza viruses with tandem bicistronic vRNA:

(A) General design of expression plasmids for transient bicistronic vRNAs coding for GFP in the mRNA-proximal, and for CAT in the mRNA-distal position. Among the functional elements indicated are the human RNA polymerase I promoter (p_{Ih}) and murine rDNA terminator (t_I) sequences, both hatched, and the 5' and 3' vRNA promoter cDNA sequences, open and closed boxes, respectively. For the vRNA-internal 3' promoter signal three variant sequences have been inserted as indicated below (positions $\overline{1}$ to $\overline{15}$ refer to 3'-terminal nucleotides in the resulting monocistronic vRNAs).

(B) CAT assays as determined relative to pHL1844 (monocistronic CAT construct) after DNA transfection of 293T cells plus helper virus infection followed by one round of progeny viral propagation on MDCK cells are indicated *below* the lanes. Relative activities of the internal promoter sequences as indicated *above* the figure refer to measurements in a monocistronic *external* location of the same promoter variants (Flick *et al.*, Promoter elements in the influenza vRNA terminal structure, RNA 2, 1046-1057 (1996)). Control clone pHL2300 contains an unrelated, non-functional sequence in the central location in an otherwise identical plasmid construct.

Fig. 3. Tandem bicistronic vRNA sup-porting an alternative mode of tran-scription and replication initiation:

An additional internal 3' promoter sequence has been inserted in between both cistrons, in a vRNA-central position. Left half: bicistronic replication and transcription leading to (proximal) GFP expression. Right half: internal initiation resulting in monocistronic replication and transcription leading to (distal) CAT expression, and causing deletion of the GFP sequence from progeny molecules.

Fig. 4. Outgrowth of promoter-up recombinant vRNA versus wildtype vRNA segments in stepwise propagation of influenza virus:

RNA polymerase I transcription of transfected pHL2969 DNA results in influenza vRNA carrying (an external) promoter-up mutant '1104', and containing 2x2 ribozyme targets in flanking positions relative to its HA coding sequence. Another HA vRNA segment in wild-type configuration and originating from infecting FPV helper virus is also present in the recombinant virus preparation, initially (lane 1; 293T lysate) in surplus amounts, but reduced and finally lost entirely in consecutive steps of propagation (lanes 2 to 4; MDCK cell lysates), and in isolated strains after pAM403 ribozyme treatment for removal of the external '1104' promoter sequence (lane 5). Determination throughout by RT-PCR analyses using a pair of primers extending across the 5' inserted target site sequence, with 435 bp representing the recombinant HA segment, and 306 bp the wild-type sequence without an inserted target site sequence.

Fig.5. pAM403 ribozyme cleavage of pHL2969 derived vRNA molecules at specific target sites inserted between an external and an internal 3' promoter sequence:

The external promoter-up ('1104') signal is used for vRNA amplification within recombinant viruses and reduction of helper virus HA vRNA (Fig. 4), while the 'switch' to an internal wild-type signal guarantees stable replication of recombinant viruses. pAM403 hammerhead ribozyme RNAs are indicated in complementary binding to their target site sequences (12 and 10 nucleotides flanking the GU'C cleavage point) by straight lines flanking a central secondary structure symbol. vRNA-internal 2x2 ribozyme targets are marked by xx (see Fig. 7).

<u>Fig. 6. Comparative cleavage analysis of model CAT vRNAs with tandem target sites in various flanking positions, by target-specific ribozymes:</u>

293T tissue culture cells have been transiently DNA-transfected either by a single-headed hammerhead ribozyme (**s**), or a double-headed (**d**), or triple-headed (**t**) ribozyme cDNA construct, all specifically designed to hybridize to a tandem dimer target site sequence inserted in flanking positions into the CAT vRNA. All ribozyme RNAs have been expressed from the same pSV2-*neo* plasmid vector, including a pSV2-*neo* control construct without an inserted ribozyme cDNA sequence (**c**). At 20 h after DNA transfection (which reached 65% yield as measured by p_{CMV}-GFP transfection in parallel of the same cell culture) the 293T cells were infected by CAT recombinant viruses carrying tandem double target sequences either only in vRNA-3' position, or in both vRNA-3' and 5' positions, or in both vRNA-3' and cRNA-3' positions. Most effective among the s, d, or t-ribozymes were double-headed constructs, acting on 2x2 targets inserted in either of the two localizations described (lanes 6 and 10).

<u>Fig. 7. Alignment of pAM424 double-headed ribozyme with one of their repetitive target sequences located within the 5' and 3' vRNA flanking regions:</u>

The superior activity of ribozymes oriented against targets located in the 3' end of vRNA molecules over those present in the 5' end instead (not shown) is in agreement with the model for influenza vRNA transcription and replication (Lamb and Krug, Orthomyxoviridae: The viruses and their replication. In 'Virology' (B.N.Fields, D.M.Knipe, P.M.Howley, R.M.Chanock, J.L.Melnick, T.P.Monath, B.Roizman, and S.E.Straus, Eds.), 3rd ed., Vol. 1, pp. 1353-1395. Lippincott-Raven, Philadelphia (1996)), according to which influenza polymerase stays attached to the 5' end of the vRNA molecule throughout the entire or even several rounds of transcription, whereas the very 3' end repeatedly, in every initiation reaction serves as the template sequence, and consequently is no longer covered by polymerase. Superiority of a double-headed over a single-headed ribozyme has been determined earlier in this laboratory (A.Menke, Anti-Influenza Ribozyme: vRNA-Spaltung und intrazelluläre Aktivität. Dissertation Universität Giessen (1997)), but the substantial increase of vRNA inactivation rates upon incorporation of tandem target sites at both ends of the vRNA

molecule instead of only one has been observed here for the first time, within that overall design.

Fig. 8: pAM424 ribozyme cleavage of resistant FPV wild-type HA vRNA and ribozyme-sensitive pHL2969-derived HA-vRNA in 293T cells infected by vHM41 after isolation from pHL2969-recombinant viral preparations. Lane 1: FPV infection of 293T cells, untreated; lane 2: FPV infection of 293T cells DNA-transfected by pAM424; lane 3: vHM41 infection of 293T cells, untreated; lane 4: vHM41 infection of 293T cells DNA-transfected by pAM424. RT-PCR analyses of purified viral progeny as in Fig. 4.

Fig. 9 Functional analysis of the influenza cRNA promoter structure:

(A) Schematic cRNA promoter ('1104') secondary structure according to the 'corkscrew' model; nucleotides involved in single or double nucleotide exchange are marked by their position.

(B) CAT analyses of 293T cell lysates after DNA transfection and FPV helper virus infection of cRNA promoter variants, in comparison to standard vRNA promoter-up mutant '1104' (pHL1844). Nucleotide substitutions divergent from the basic '1104' structure as present in pHL2583 or pHL2721 (see above) are indicated above the lanes, positions $\bar{3}$ or $\bar{8}$ as marked by a bar refer to cRNA positions counted from the 3' end. Relative CAT activities are marked below the lanes.

Fig.10 Functional analysis of the vRNA and cRNA promoter in ambisense arrangement:

(A) Sequence organisation of the ambisense promoter cDNA construct carrying T_6/A_6 elements adjacent to the terminal sequence, and secondary structure predictions for the resulting cRNA and vRNA promoter signal.

(B) CAT expression data obtained from the cell lysates of 293T cell after plasmid DNA transfection and FPV infection, and (C) from cell lysates of MDCK cells after one step of viral passage. Indicated above the lanes are promoter/gene conjunctions: v = vRNA promoter; c = cRNA promoter.

Fig.11 Basepair substitutions according to the vRNA 'corkscrew' structure:

(A) 'Corkscrew' conformation of the vRNA promoter drawn against a schematic indication of interacting tripartite viral polymerase. Paired positions exchanged in individual experiments are indicated by numbers, nucleotides $\bar{3}$ or $\bar{8}$ are counted from the 3' end. pHL2024 containing promoter-up mutation '1104' is used as the reference construct (=100%) in all of the CAT assays, while pHL2428 represents the wild-type promoter structure.

(B) CAT expression data obtained after one step of viral passage in MDCK undiluted, and 50 ~~fold diluted.~~

Fig. 12: Flow-chart of the isolation procedure for an ambisense recombinant influenza virus.

Fig. 13 Immuno-electron microscopy of purified influenza FPV/CSFV-E2-HA virions:

Recombinant viruses exposing the foreign glycoprotein CSFV-E2 in their envelopes, which has been fused onto the HA anchor domain, are marked by anti-E2 monospecific antibody and by secondary gold-labelled (5nm) goat antibody. Recombinant viruses (16%) are present together with their FPV helper viruses.

Fig. 14: pHL2969; the exact sequence of the 4930 bps circular DNA is shown in SEQ. ID NO:1.

Fig. 15: pAM403; the exact sequence of the 5811 bps circular DNA is shown in SEQ. ID NO:2.

Fig. 16: pAM424; the exact sequence of the 5860 bps circular DNA is shown in SEQ. ID NO:5.

Fig. 17: pHL2507; the exact sequence of the 4610 bps circular DNA is shown in SEQ. ID NO:6.

Fig. 18: pHL2583; the exact sequence of the 3558 bps circular DNA is shown in SEQ. ID NO:7.

Fig. 19: pHL2989; the exact sequence of the 4343 bps circular DNA is shown in SEQ. ID NO:8.

Fig. 20: pHL1920; the exact sequence of the 3888 bps circular DNA is shown in SEQ. ID NO:9.

Hence, the present invention is based on two surprising findings, namely

1. influenza virus promoters are active when present internally in a gene;

2. the so-called cRNA, thought to be an intermediate in replication can be turned into a protein-encoding RNA by equipping it with a variant influenza virus promoter, described in the present invention.

These two observations were used to make ambisense constructs. This allows to package an additional, foreign gene into influenza virus particles. Such particles were made previously, by other methods, but proved to be unstable, and therefore useless. For use as a vaccine for example, a helper virus would have been needed as a stabilizer. Stabilization in the present invention is achieved by several means. These include the "balancing" of one of the two promoters in the ambisense biscistronic genetic construct with seven other vRNA wildtype promoters, while the additional promoter is used for high-rate expression of the foreign gene at various levels.

Thus, the present invention provides a system for expression of foreign proteins in higher eukaryotic systems. One system in particular is interesting, namely embryonated chicken eggs, as it allows cost-effective production in an automatable way (as used by most flu vaccine producers). The reason that this process is now possible, is that the foreign protein is part of a stable, engineered influenza virus particle. The virus can be designed also to rapidly monitor process improvements.

An excellent use is of course the use of the construct as a vaccine. The influenza virus particle is immunogenic and can now be equipped with foreign antigens, enabling for example the design and production of hepatitis C virus and HIV vaccines, but also of tumor vaccines. As the present invention shows, the foreign antigenic surface glycoprotein is "fused" to a C-

terminal segment of influenza HA, and the antigen then is presented at the surface of influenza virus particles. In addition, these vaccines can now be made in the way standard flu vaccines are made, i.e., in embryonated chicken eggs.

SEQUENCE LISTING

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aaaggatcta ggtgaagatc ctttttgata atctcatgac caaaatccct taacgtgagt 3600
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tagcaccgcc tacatacctc gctctgctaa tcctgttacc agtggctgct gccagtggcg 3900

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gaaacgcctg gtatctttat agtcctgtcg ggtttcgcca cctctgactt gagcgtcgat 4200
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attcattaat gcaggtcacg atc 4343

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<211> 3888
<212> DNA
<213> Influenza virus

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aaaaaaaaac aaagtttcgc ccggagtact ggtcgacctc cgaagttggg ggggagtaga 180
aacagggtag ataatcactc actgagtgac atccacatcg cgagcgcgcg taatacgact 240
cactataggg cgaattgggt accgggcccc ccctcgaggt cgacggtatc gataagcttc 300
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tccggaattc cgtatggcaa tgaaagacgg tgagctggtg atatgggata gtgttcaccc 600
ttgttacacc gttttccatg agcaaactga aacgttttca tcgctctgga gtgaatacca 660
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cctggcctat ttccctaaag ggtttattga gaatatgttt ttcgtctcag ccaatccctg 780
ggtgagtttc accagttttg atttaaacgt ggccaatatg gacaacttct tcgcccccgt 840
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tacggctaca ctagaaggac agtatttggt atctgcgctc tgctgaagcc agttaccttc 2220
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cgaaaactct caaggatctt accgctgttg agatccagtt cgatgtaacc cactcgtgca 3240
cccaactgat cttcagcatc ttttactttc accagcgttt ctgggtgagc aaaaacagga 3300
aggcaaaatg ccgcaaaaaa gggaataagg gcgacacgga aatgttgaat actcatactc 3360
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tttgaatgta tttagaaaaa taaacaaaag agtttgtaga aacgcaaaaa ggccatccgt 3480
caggatggcc ttctgcttaa tttgatgcct ggcagtttat ggcgggcgtc ctgcccgcca 3540
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gagagcgttc accgacaaac aacagataaa acgaaaggcc cagtctttcg actgagcctt 3660
tcgttttatt tgatgcctgg cagttcccta ctctcgcatg gggagacccc acactaccat 3720
cggcgctacg gcgtttcact tctgagttcg gcatggggtc aggtgggacc accgcgctac 3780
tgccgccagg caaattctgt tttatcagac cgcttctgcg ttctgattta atctgtatca 3840
ggctgaaaat cttctctcat ccgccaaaac agaagctagc ggccgatc 3888

Claims

1. A recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus; wherein
(a) at least one of the regular viral RNA segments has been exchanged for a vRNA encoding a foreign gene; and/or
~~(b) at least one of the regular viral RNA segments is an ambisense RNA molecule, containing~~ one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement.

2. The recombinant virus according to claim 1, wherein in the ambisense RNA molecule said foreign recombinant gene is covalently bound to one of the viral genes, while the original vRNA segment coding for the same gene is deleted from the recombinant virus by way of specific ribozyme cleavage.

3. The recombinant virus according to claims 1 and 2 in which one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged into foreign glycoprotein(s) or into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

4. The recombinant virus according to claims 1 to 3, in which the terminal viral RNA sequences, which are active as the promoter signal, have been varied by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence.

5. The recombinant virus according to claims 1 to 4, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus.

6. The recombinant virus according to claims 1 to 4, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of (abortively) infected cells, for stimulation of a B cell and /or T cell response.

7. A method for the production of recombinant influenza viruses as defined in claims 1 to 6 comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in antisense, sense or ambisense design,

(b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA segments, and

(c) thereafter selective vRNA inactivation through ribozyme cleavage.

8. A method of constructing influenza carrier strains carrying one or more ribozyme target sites (of type one) in vRNA flanking positions comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, carrying two different 3' promoter sequences in tandem, which are separated by a second type of ribozyme target sequence, and which carry the said internal ribozyme target sites of type one;

(b) followed by infection of an influenza wildtype strain;

(c) thereafter amplification through simple steps of viral propagation; and

(d) finally isolation through removal of their external 3' promoter sequence by ribozyme cleavage through infection of cells expressing ribozyme type 2, followed by plaque purification.

9. A ribozyme-sensitive influenza carrier strain obtainable by the method of claim 8.

10. A pharmaceutical composition comprising a recombinant virus according to claims 1 to 6.

11. Use of a recombinant virus according to claims 1 to 6 for preparing a medicament for vaccination purposes.

12. The use according to claim 11, wherein the medicament
(a) is suitable against influenza and/or against other infections;
(b) is present in form of inactivated preparations; and/or
(c) is present in form of live recombinant (attenuated) viruses.

13. Use of a recombinant virus according to claims 1 to 6 as vector systems in somatic gene therapy, for transfer and expression of foreign genes into cells (abortively) infected by such viruses, either in *ex vivo* or *in vivo* application schemes.

14. A method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus according to claims 1 to 6 as expression vector.

15. The method of claim 14, wherein the production is performed in cell culture cells or in fertilized chicken eggs.

FIG. 1

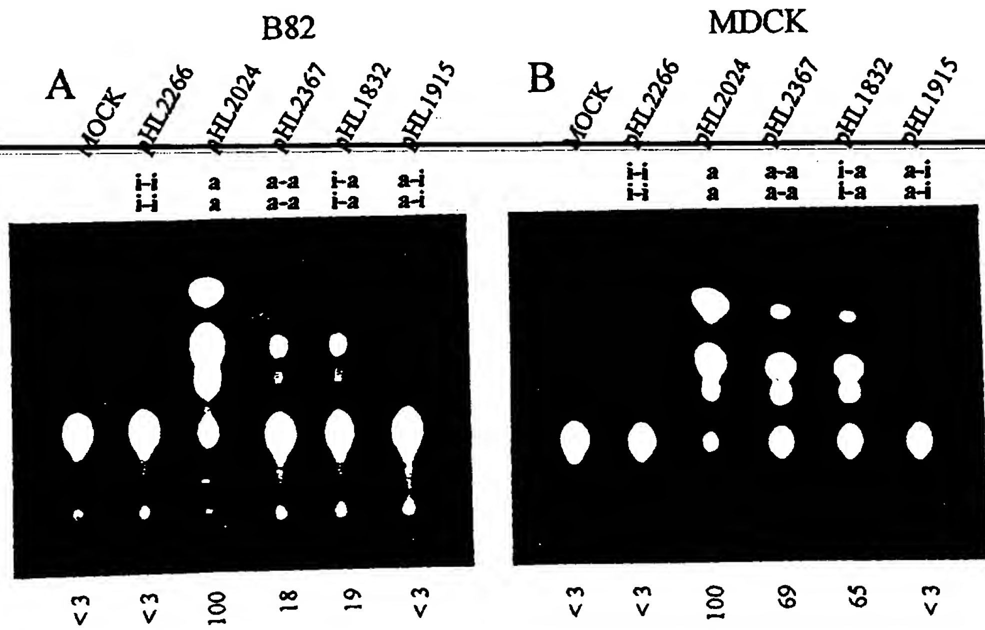

B82
MDCK
A
B
MOCK
pHL2266
pHL2024
pHL2367
pHL1832
pHL1915
i-i i-i
a a
a-a a-a
i-a i-a
a-i a-i
<3
<3
100
18
19
<3
<3
<3
100
69
65
<3

FIG. 2

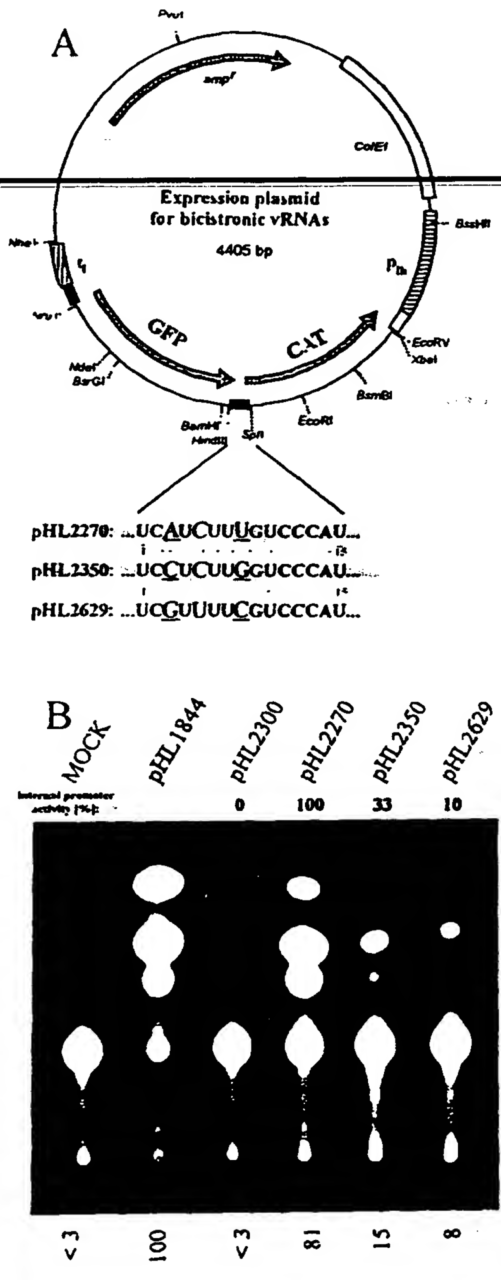

A
Pvul
amp^r
ColE1
Expression plasmid
for bicistronic vRNAs
4405 bp
BssHII
Nhe I
GFP
CAT
EcoRV
XbaI
NdeI
BsrGI
BsmBI
EcoRI
BamHI
HindIII
SpII
pHL2270: ...UCAUCUUUGUCCCAU...
pHL2350: ...UCCUCUUGGUCCCAU...
pHL2629: ...UCGUUUUCGUCCCAU...
B
MOCK
pHL1844
pHL2300
pHL2270
pHL2350
pHL2629
Internal promoter activity [%]:
0
100
33
10
<3
100
<3
81
15
8

FIG. 3

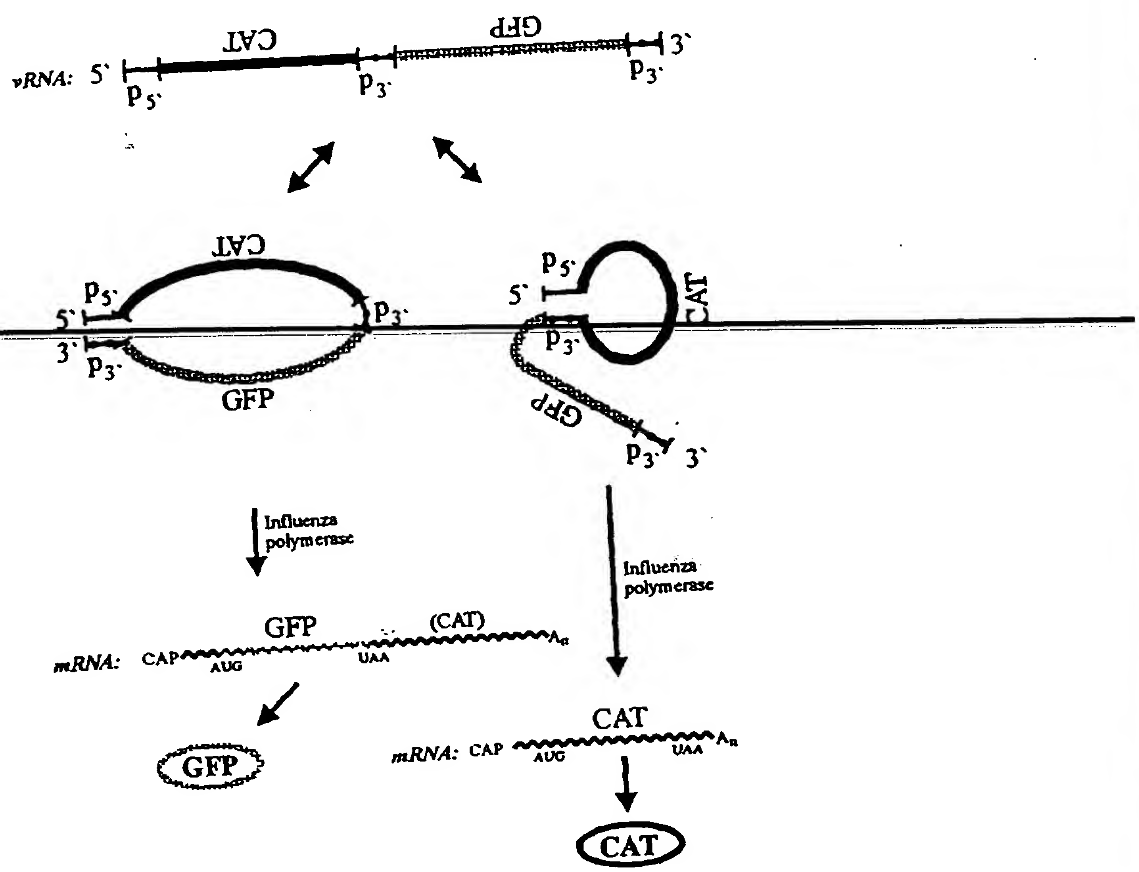

vRNA: 5'
CAT
GFP
3'
P5'
P3'
P3'
CAT
P5'
5'
P3'
3'
P3'
GFP
P5'
5'
CAT
P3'
GFP
P3'
3'
Influenza polymerase
Influenza polymerase
GFP
(CAT)
mRNA: CAP
AUG
UAA
An
GFP
CAT
mRNA: CAP
AUG
UAA
An
CAT

FIG. 4

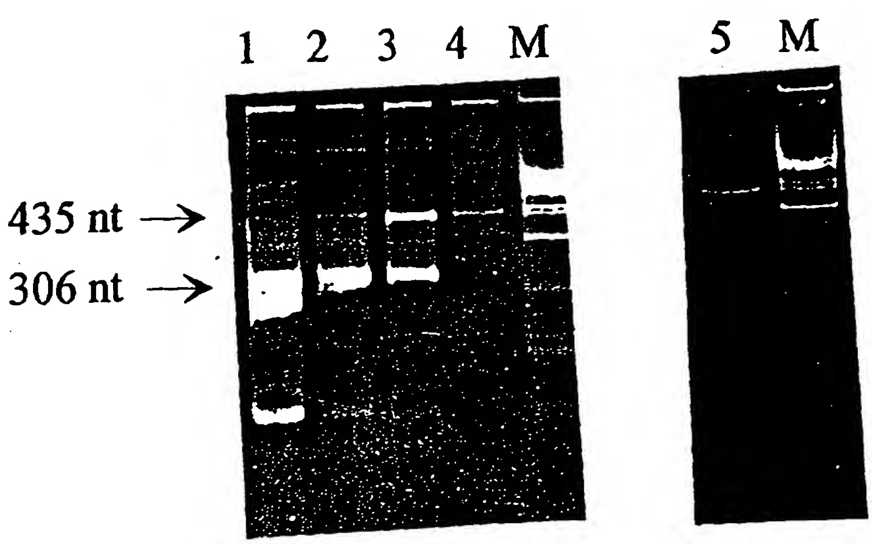

1
2
3
4
M
5
M
435 nt
306 nt

FIG. 5

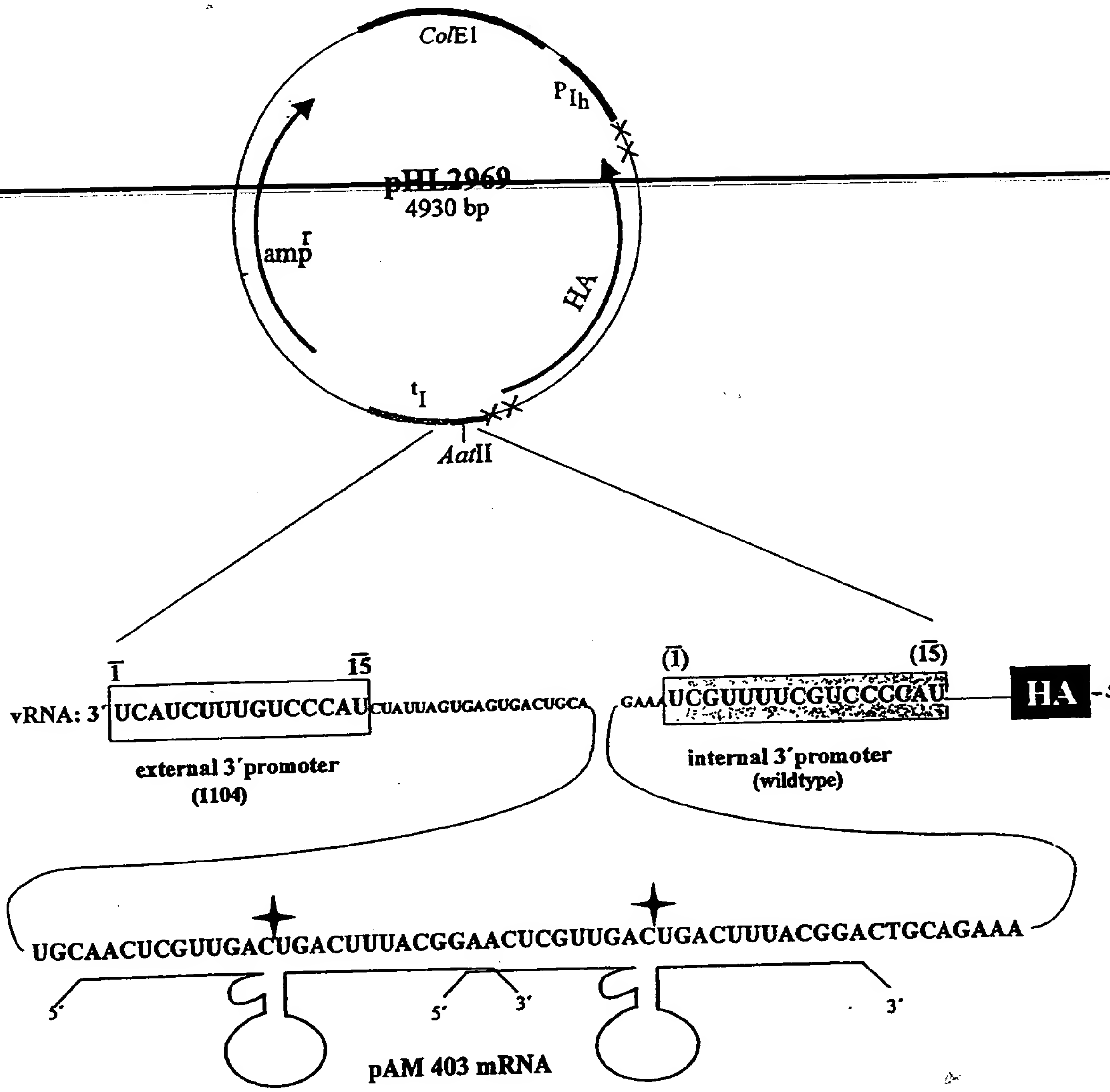

ColE1
P_{Ih}
pHL2969
4930 bp
amp^r
HA
t_I
AatII
1
15
vRNA: 3' UCAUCUUUGUCCCAU CUAUUAGUGAGUGACUGCA
external 3'promoter
(1104)
(1)
(15)
GAA UCGUUUUCGUCCCCAU
HA
-5
internal 3'promoter
(wildtype)
UGCAACUCGUUGACUGACUUUACGGAACUCGUUGACUGACUUUACGGACTGCAGAAA
5'
5'
3'
3'
pAM 403 mRNA

FIG. 6

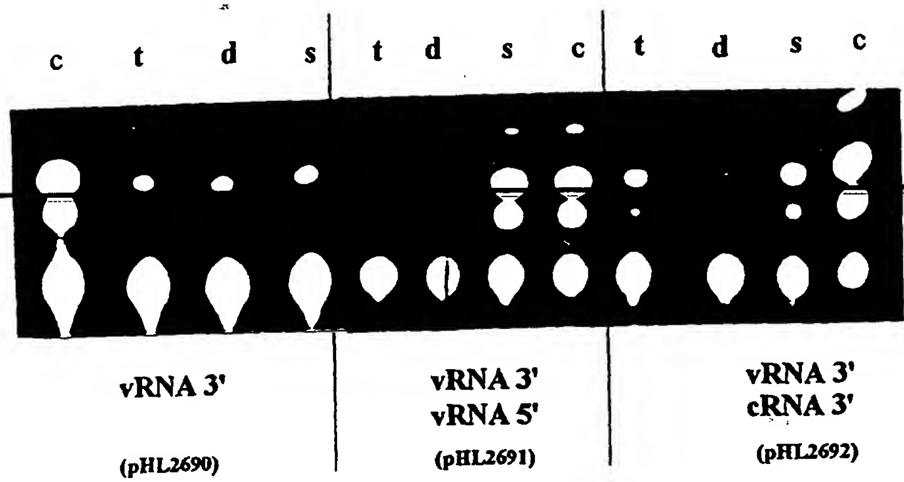

c t d s t d s c t d s c
vRNA 3'
(pHL2690)
vRNA 3'
vRNA 5'
(pHL2691)
vRNA 3'
cRNA 3'
(pHL2692)

FIG. 7

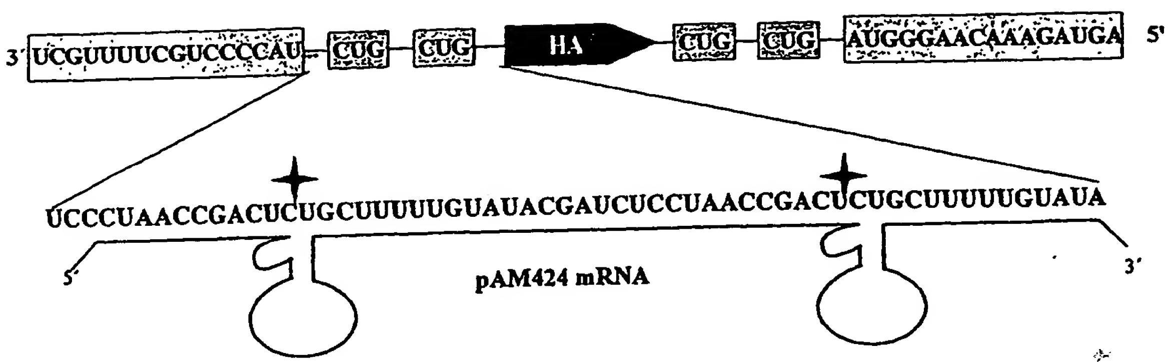

3' UCGUUUUCGUCCCCAU
CUG
CUG
HA
CUG
CUG
AUGGGAACAAAGAUGA 5'
UCCCUAACCGACUCUGCUUUUUGUAUACGAUCUCCUAACCGACUCUGCUUUUUGUAUA
5'
3'
pAM424 mRNA

FIG. 8

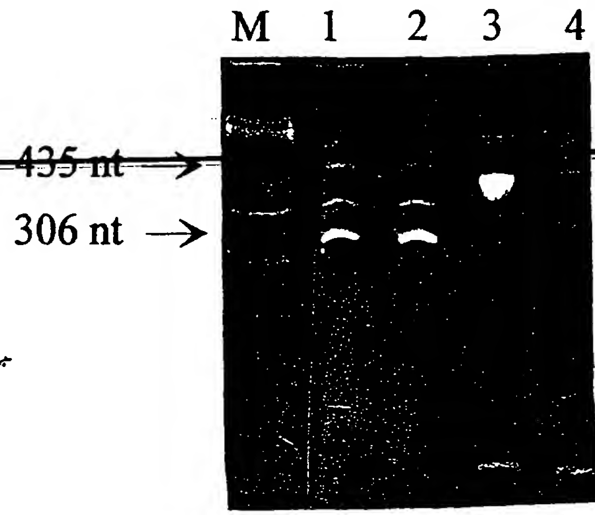
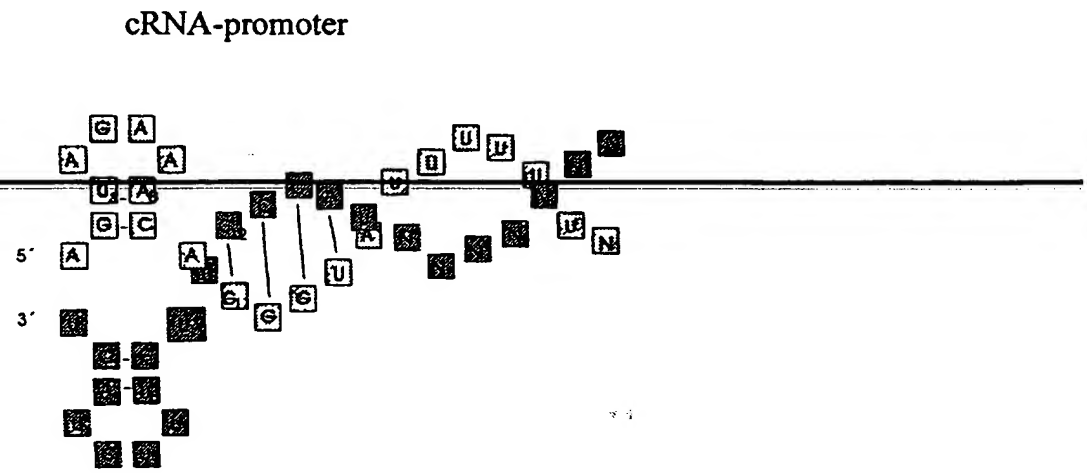


FIG. 9


cRNA-promoter
5'
3'

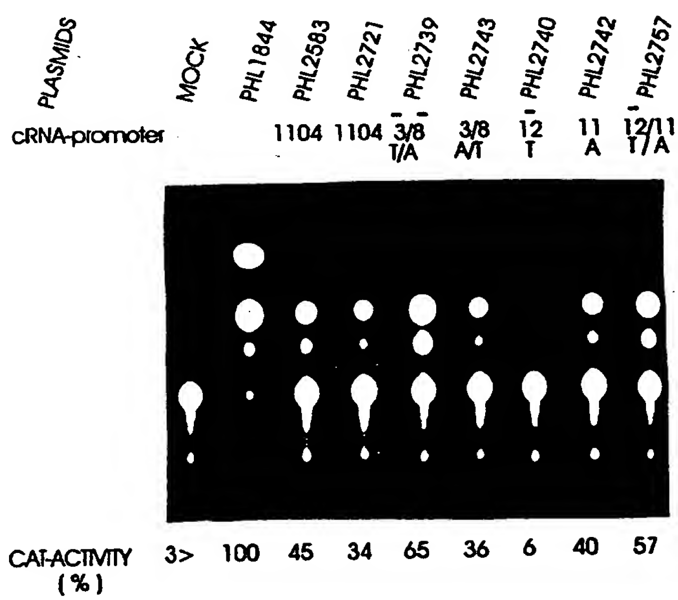

PLASMIDS
MOCK
PHL1844
PHL2583
PHL2721
PHL2739
PHL2743
PHL2740
PHL2742
PHL2757
cRNA-promoter
1104 1104 3/8 T/A 3/8 A/T 12 T 11 A 12/11 T/A
CAT-ACTIVITY (%)
3> 100 45 34 65 36 6 40 57

FIG. 10

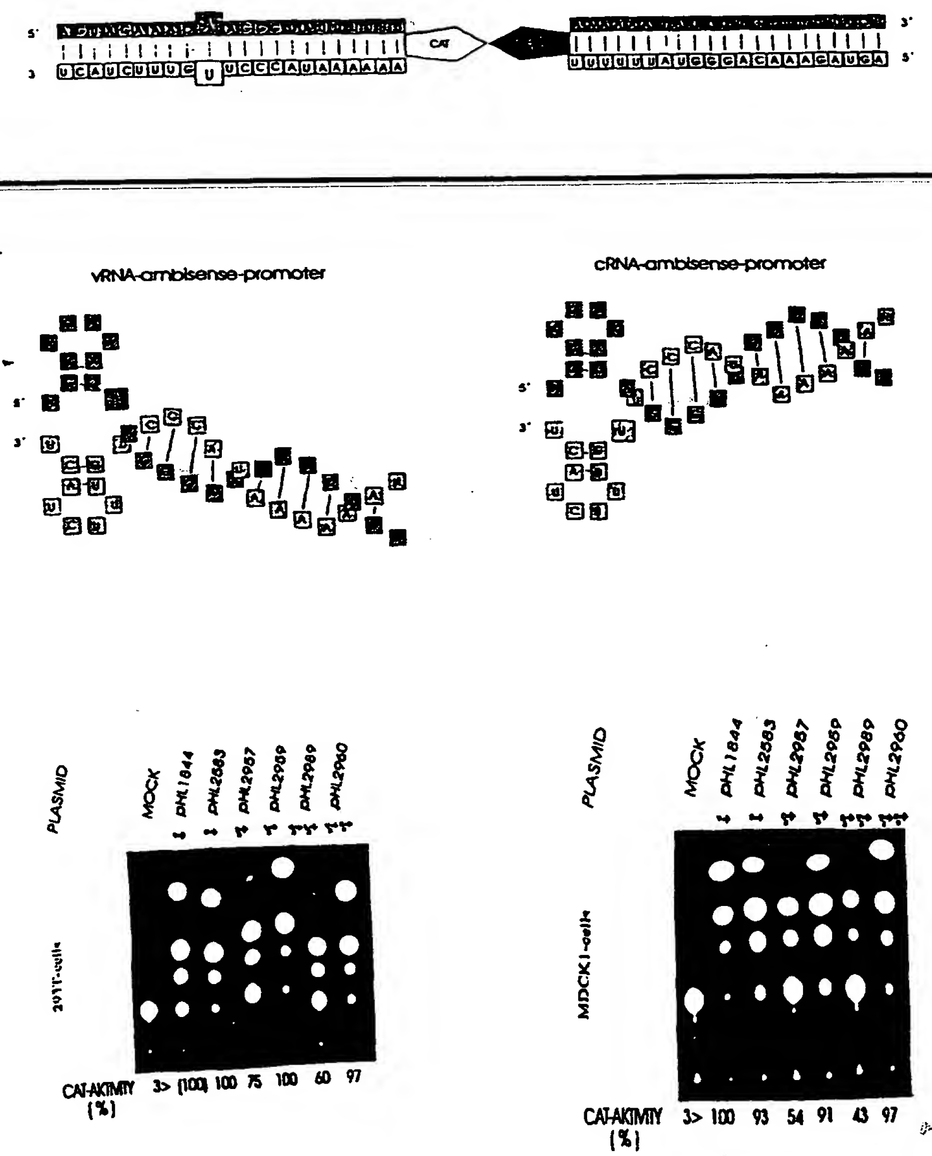
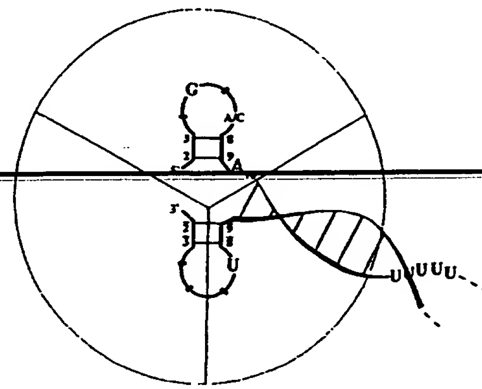

CAT
vRNA-ambisense-promoter
cRNA-ambisense-promoter
PLASMID
MOCK
PHL1844
PHL2583
PHL2957
PHL2969
PHL2989
PHL2960
293T-cells
CAT-AKTIVITY (%) 3> (100) 100 75 100 60 97
MDCK1-cells
CAT-AKTIVITY (%) 3> 100 93 54 91 43 97

FIG. 11

A



bp-variant / position	G - C	A - U	C - G	U - A
2 - 9	pHL2024 100%	pHL1921 41%	pHL2003 < 3%	pHL2004 < 3%
3 - 8	pHL2002 30%	pHL1920 121%	pHL1148 39%	pHL2024 100%
$\bar{2}$ - $\bar{9}$	pHL1945 11%	pHL1946 30%	pHL2024 100%	pHL1923 28%
$\bar{3}$ - $\bar{8}$	pHL2428 6%	pHL2024 100%	pHL1948 33%	pHL1922 97%

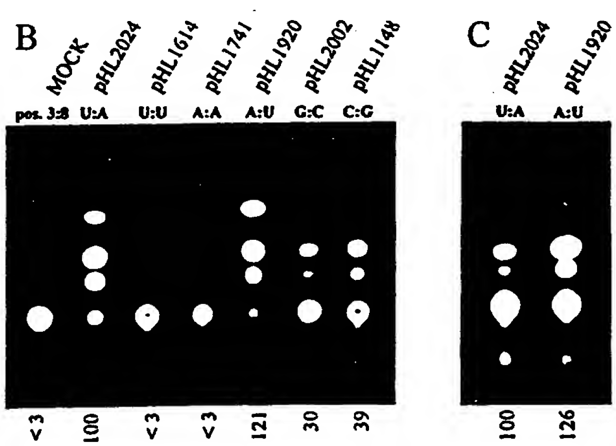
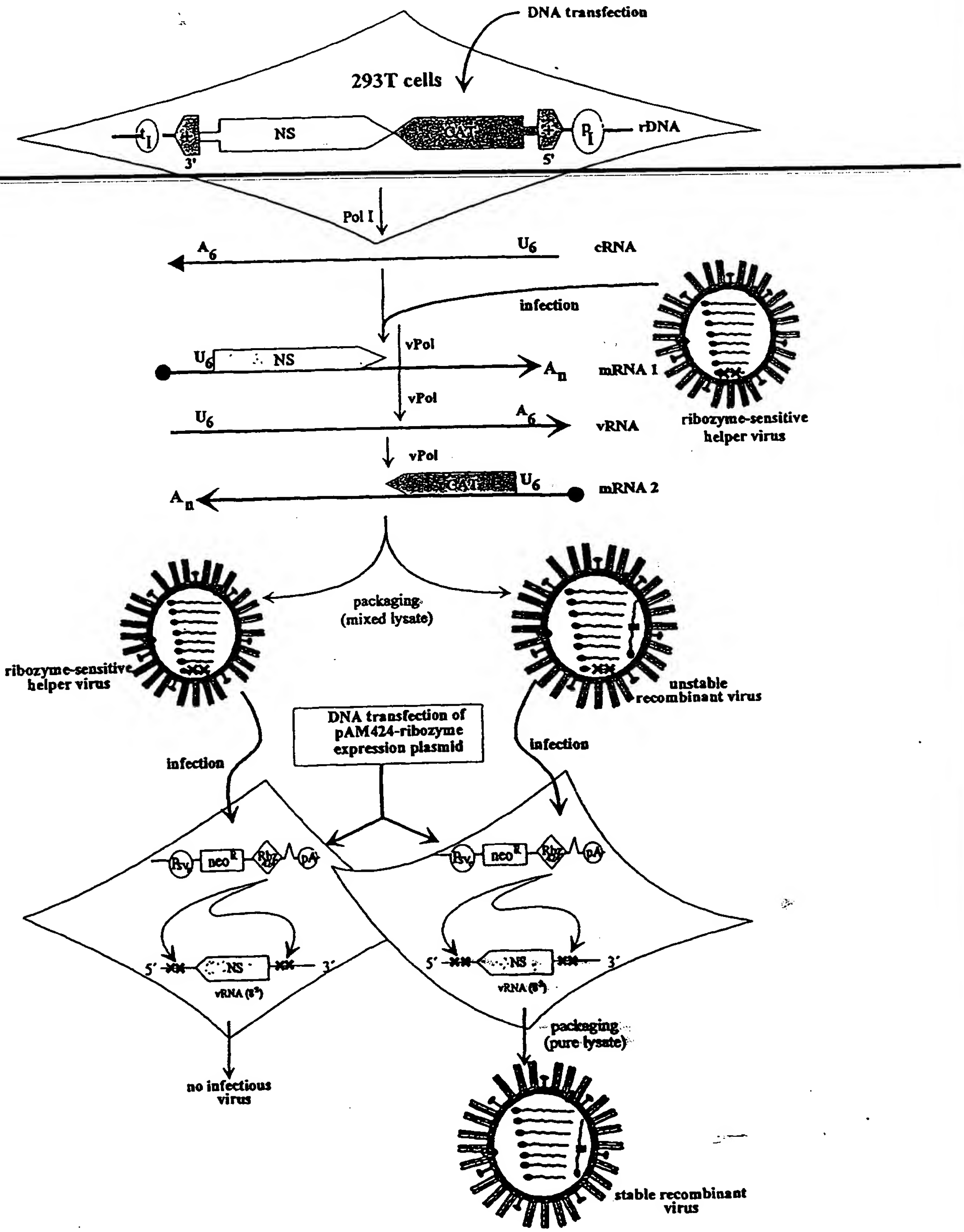


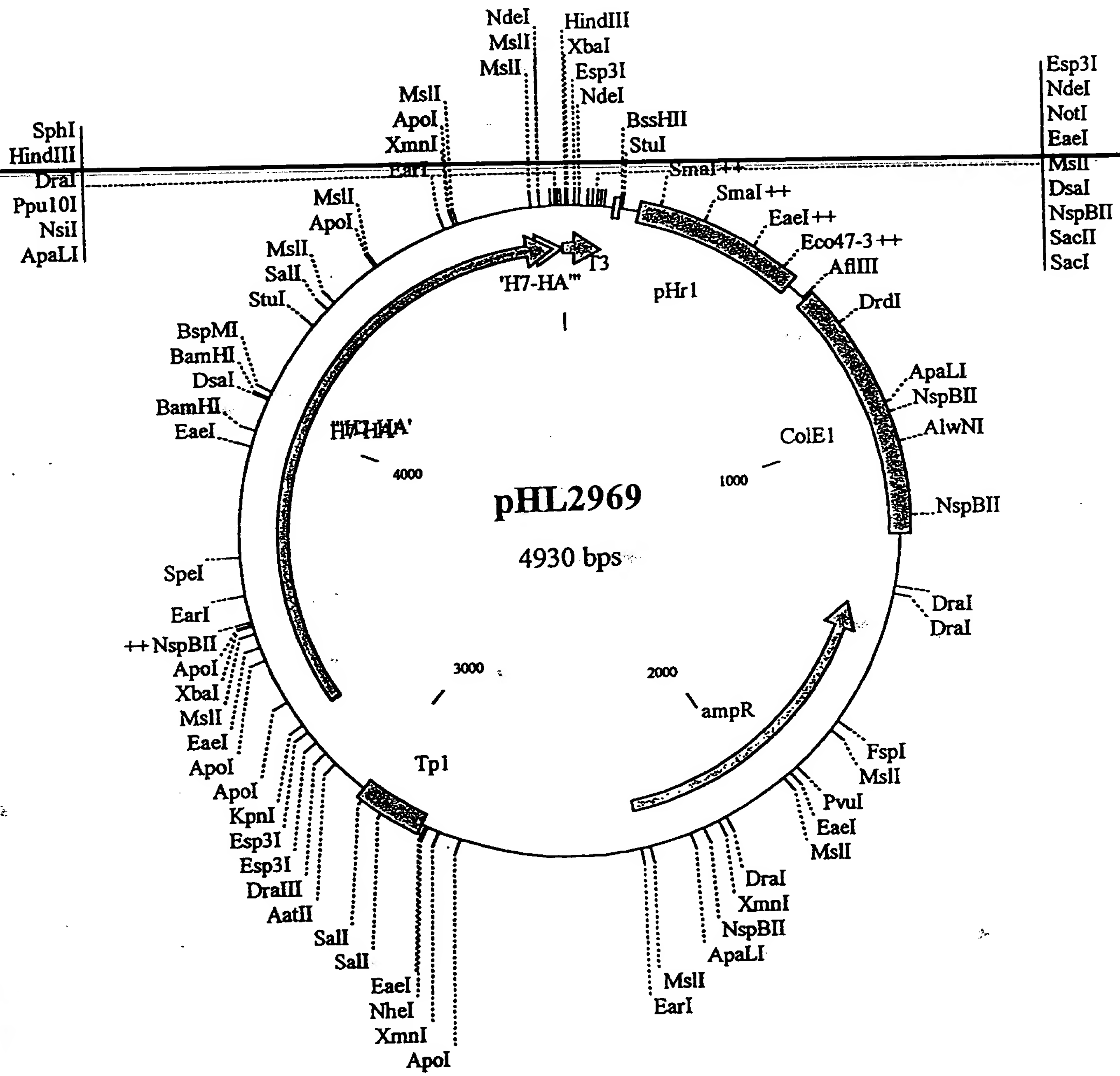
FIG. 12



DNA transfection
293T cells
NS
CAT
3'
5'
rDNA
Pol I
A_6
U_6
cRNA
infection
vPol
U_6 NS
A_n
mRNA 1
vPol
U_6
A_6
vRNA
ribozyme-sensitive helper virus
vPol
CAT
U_6
A_n
mRNA 2
packaging (mixed lysate)
ribozyme-sensitive helper virus
unstable recombinant virus
DNA transfection of pAM424-ribozyme expression plasmid
infection
infection
neo^R
pA
5'
NS
3'
no infectious virus
packaging (pure lysate)
stable recombinant virus

FIG. 13

FIG. 14



pHL2969
4930 bps
'H7-HA'''
T3
pHr1
ColE1
ampR
Tp1
'H7-HA'
1000
2000
3000
4000
NdeI
MslI
MslI
HindIII
XbaI
Esp3I
NdeI
BssHII
StuI
SmaI ++
SmaI ++
EaeI ++
Eco47-3 ++
AflIII
DrdI
ApaLI
NspBII
AlwNI
NspBII
DraI
DraI
FspI
MslI
PvuI
EaeI
MslI
DraI
XmnI
NspBII
ApaLI
MslI
EarI
EaeI
NheI
XmnI
ApoI
SalI
SalI
AatII
DraIII
Esp3I
Esp3I
KpnI
ApoI
ApoI
EaeI
MslI
XbaI
ApoI
++NspBII
EarI
SpeI
EaeI
BamHI
DsaI
BamHI
BspMI
StuI
SalI
MslI
ApoI
MslI
EarI
XmnI
ApoI
MslI
SphI
HindIII
DraI
Ppu10I
NsiI
ApaLI
Esp3I
NdeI
NotI
EaeI
MslI
DsaI
NspBII
SacII
SacI

FIG. 15

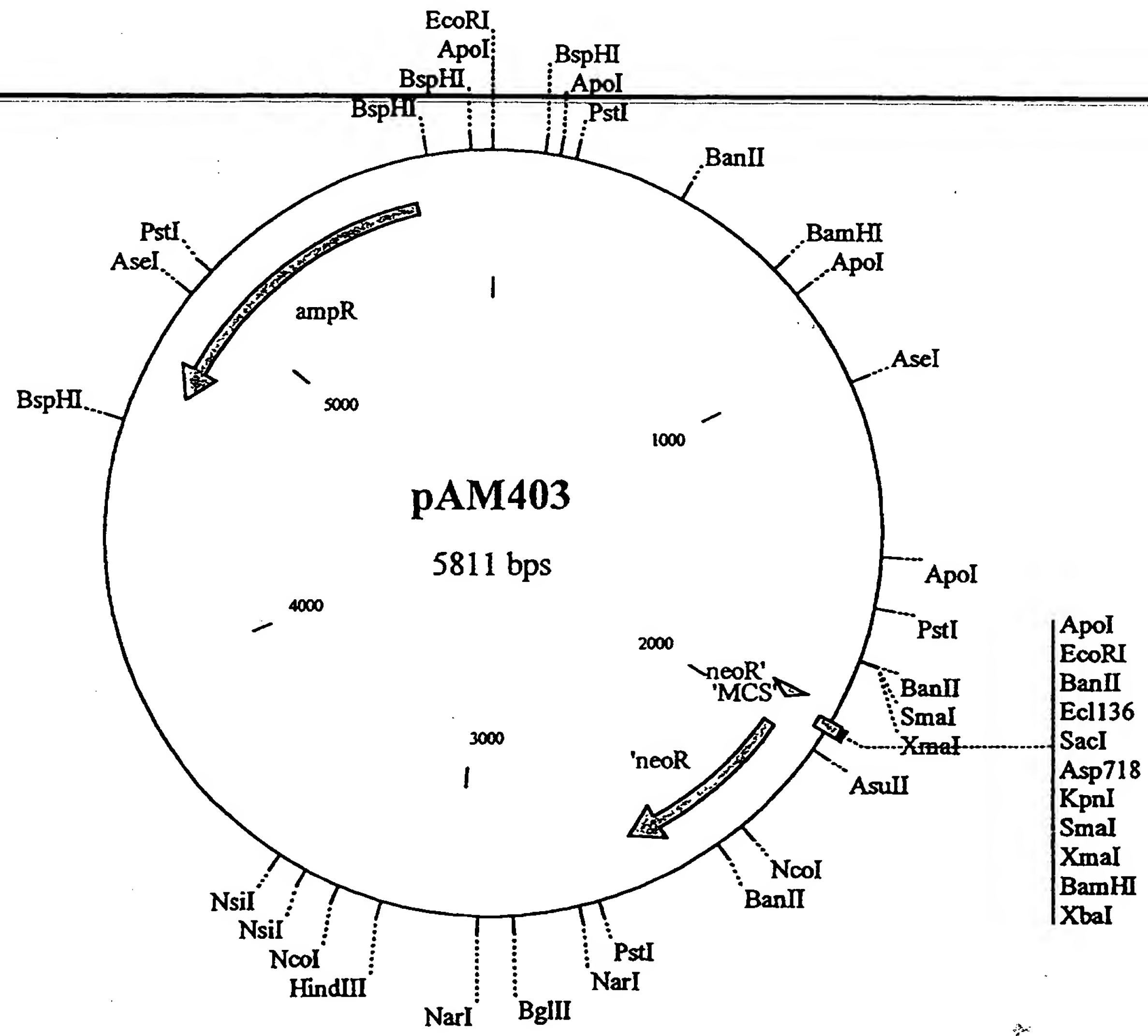

EcoRI
ApoI
BspHI
BspHI
BspHI
ApoI
PstI
BanII
BamHI
ApoI
AseI
ApoI
PstI
BanII
SmaI
XmaI
ApoI
EcoRI
BanII
Ecl136
SacI
Asp718
KpnI
SmaI
XmaI
BamHI
XbaI
AsuII
NcoI
BanII
PstI
NarI
BglII
NarI
HindIII
NcoI
NsiI
NsiI
BspHI
AseI
PstI
ampR
5000
1000
pAM403
5811 bps
4000
2000
neoR'
'MCS'
3000
'neoR

FIG. 16

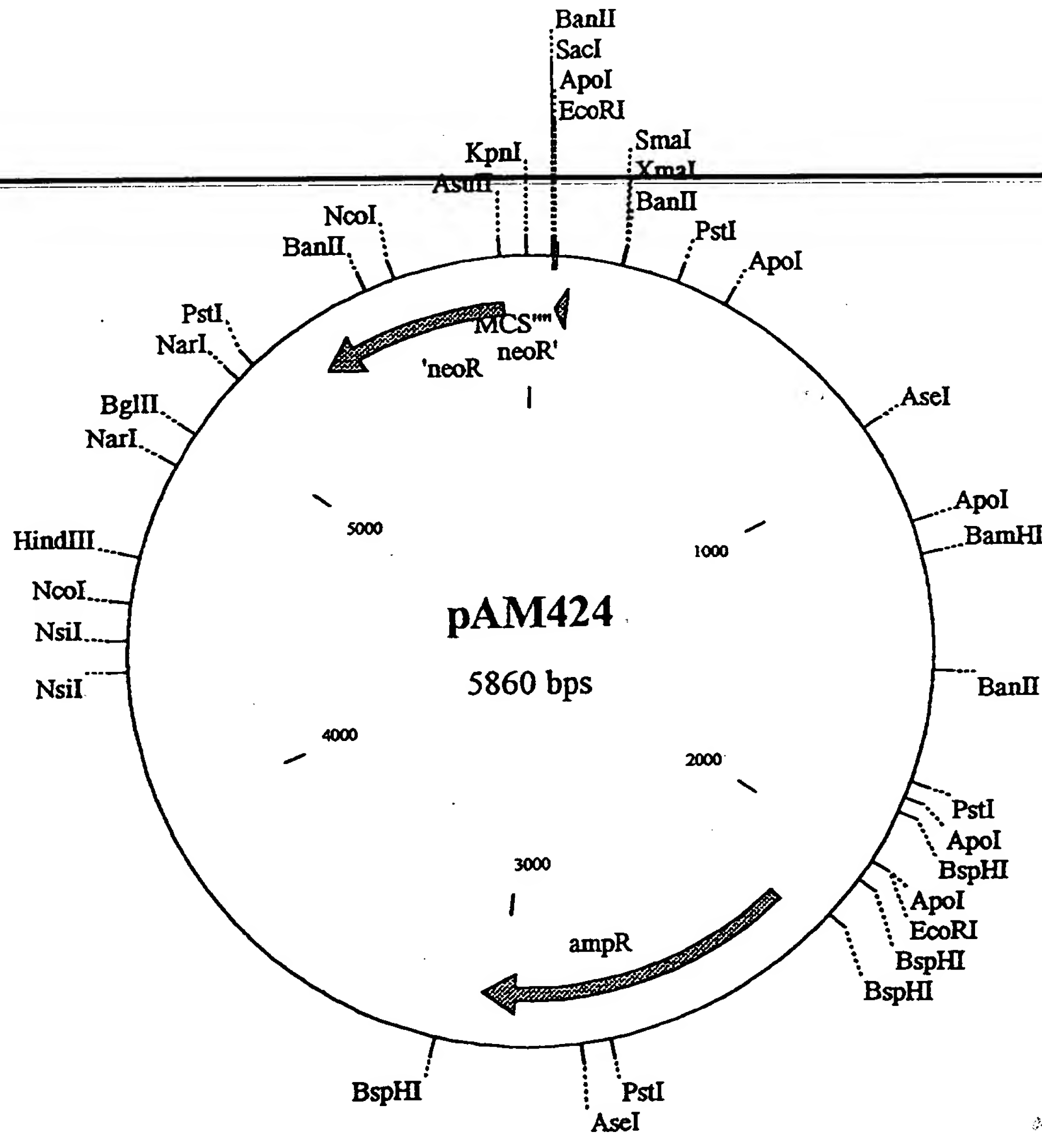

BanII
SacI
ApoI
EcoRI
SmaI
XmaI
BanII
PstI
ApoI
KpnI
AsuII
NcoI
BanII
PstI
NarI
BglII
NarI
HindIII
NcoI
NsiI
NsiI
MCS
neoR'
'neoR
AseI
ApoI
BamHI
BanII
PstI
ApoI
BspHI
ApoI
EcoRI
BspHI
BspHI
PstI
AseI
BspHI
ampR
1000
2000
3000
4000
5000
pAM424
5860 bps

FIG. 17

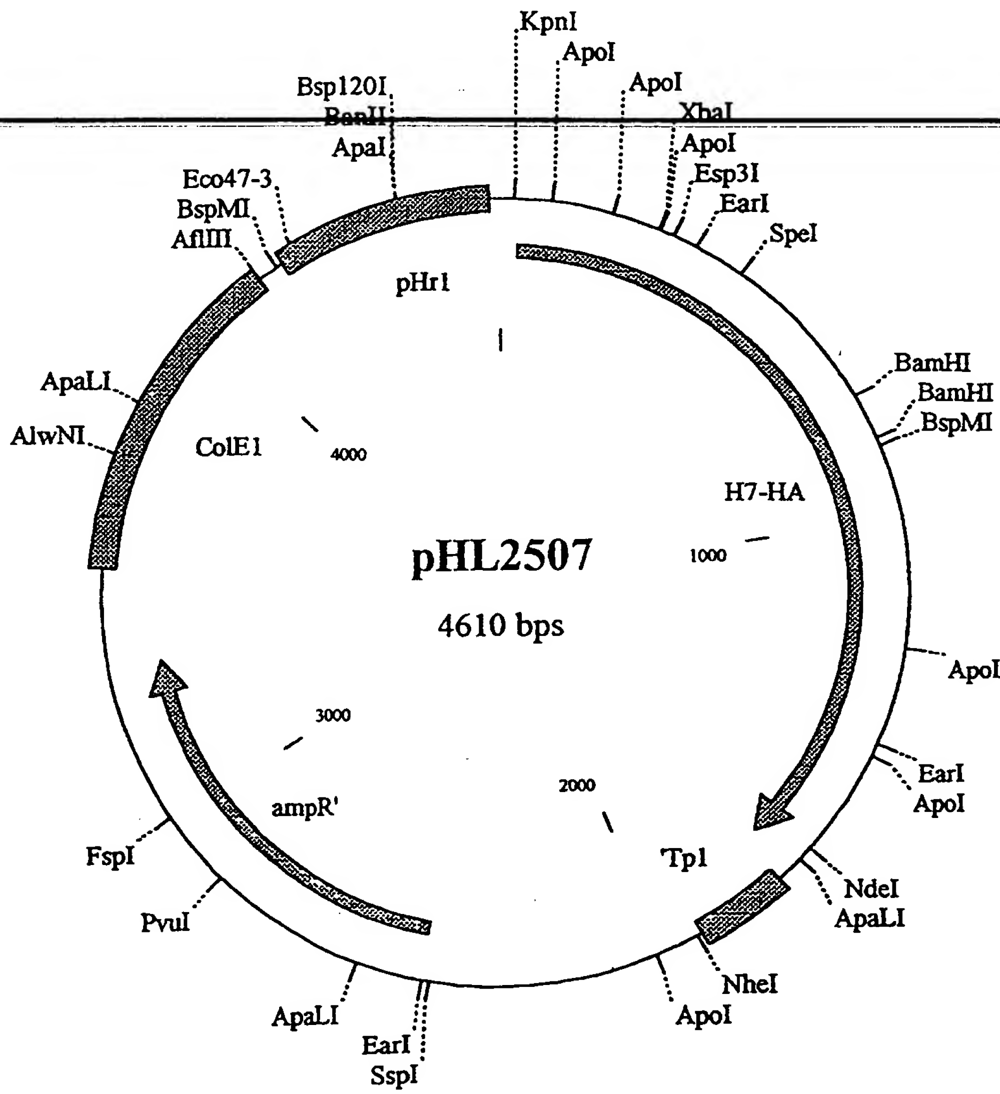

KpnI
ApoI
ApoI
XbaI
ApoI
Esp3I
EarI
SpeI
Bsp120I
BanII
ApaI
Eco47-3
BspMI
AflIII
pHr1
ApaLI
AlwNI
ColE1
4000
BamHI
BamHI
BspMI
H7-HA
1000
pHL2507
4610 bps
ApoI
3000
2000
EarI
ApoI
ampR'
Tp1
NdeI
ApaLI
FspI
PvuI
NheI
ApoI
ApaLI
EarI
SspI

FIG. 18

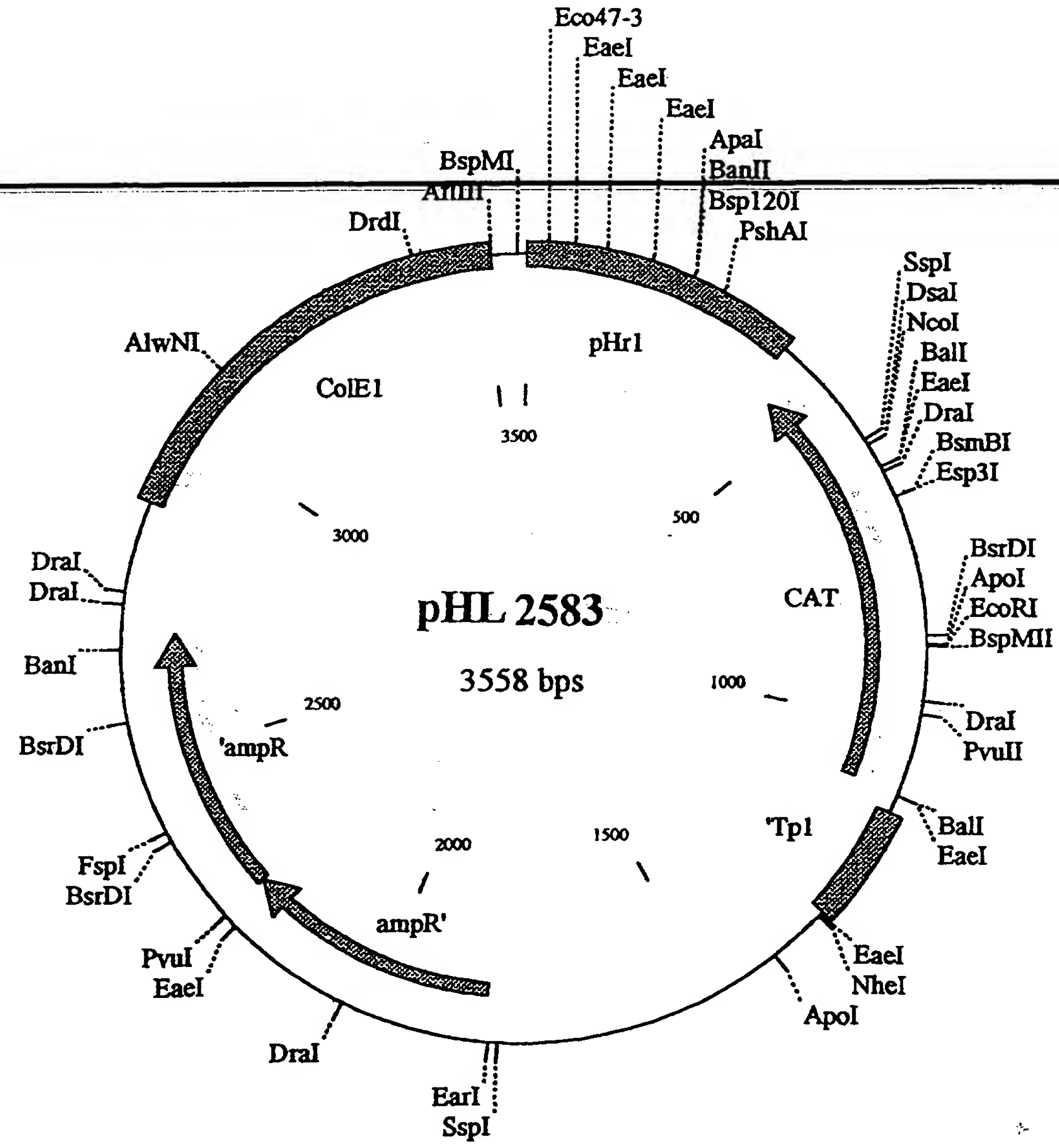
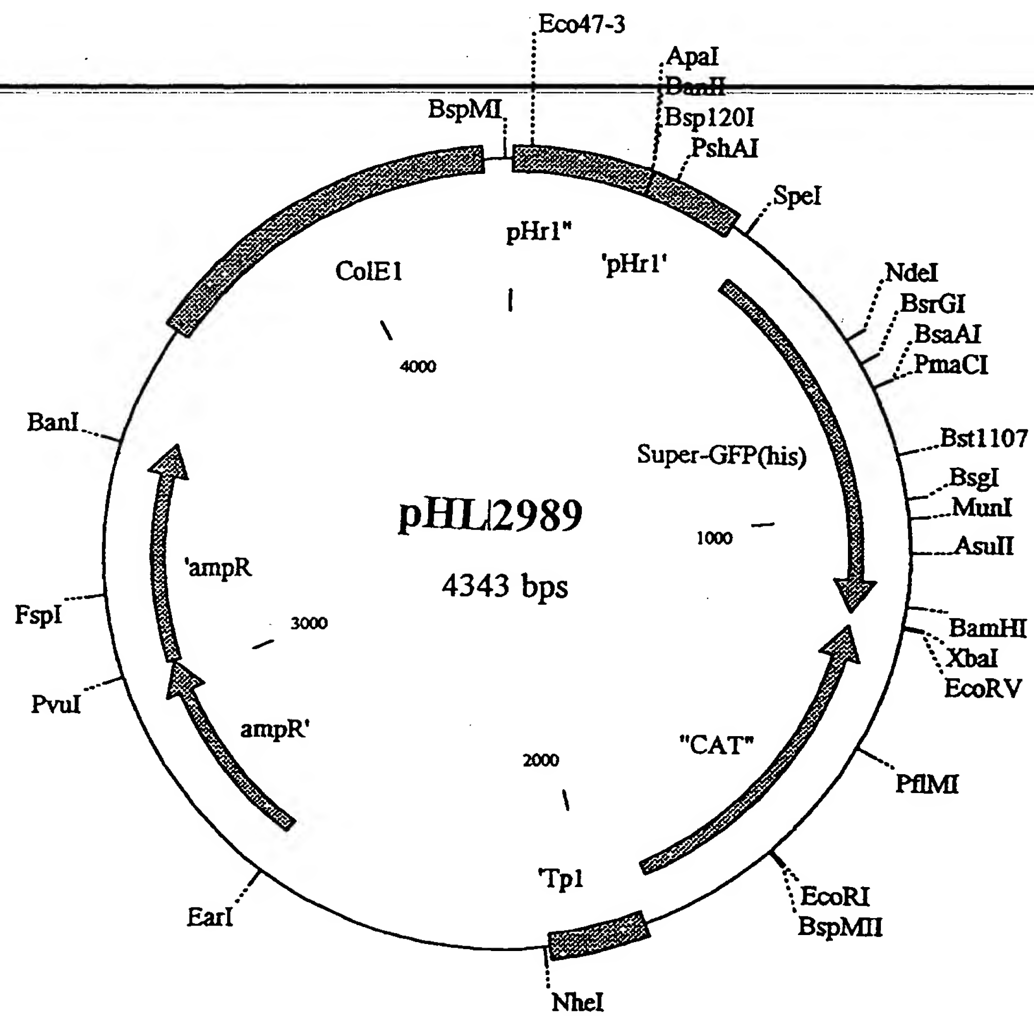
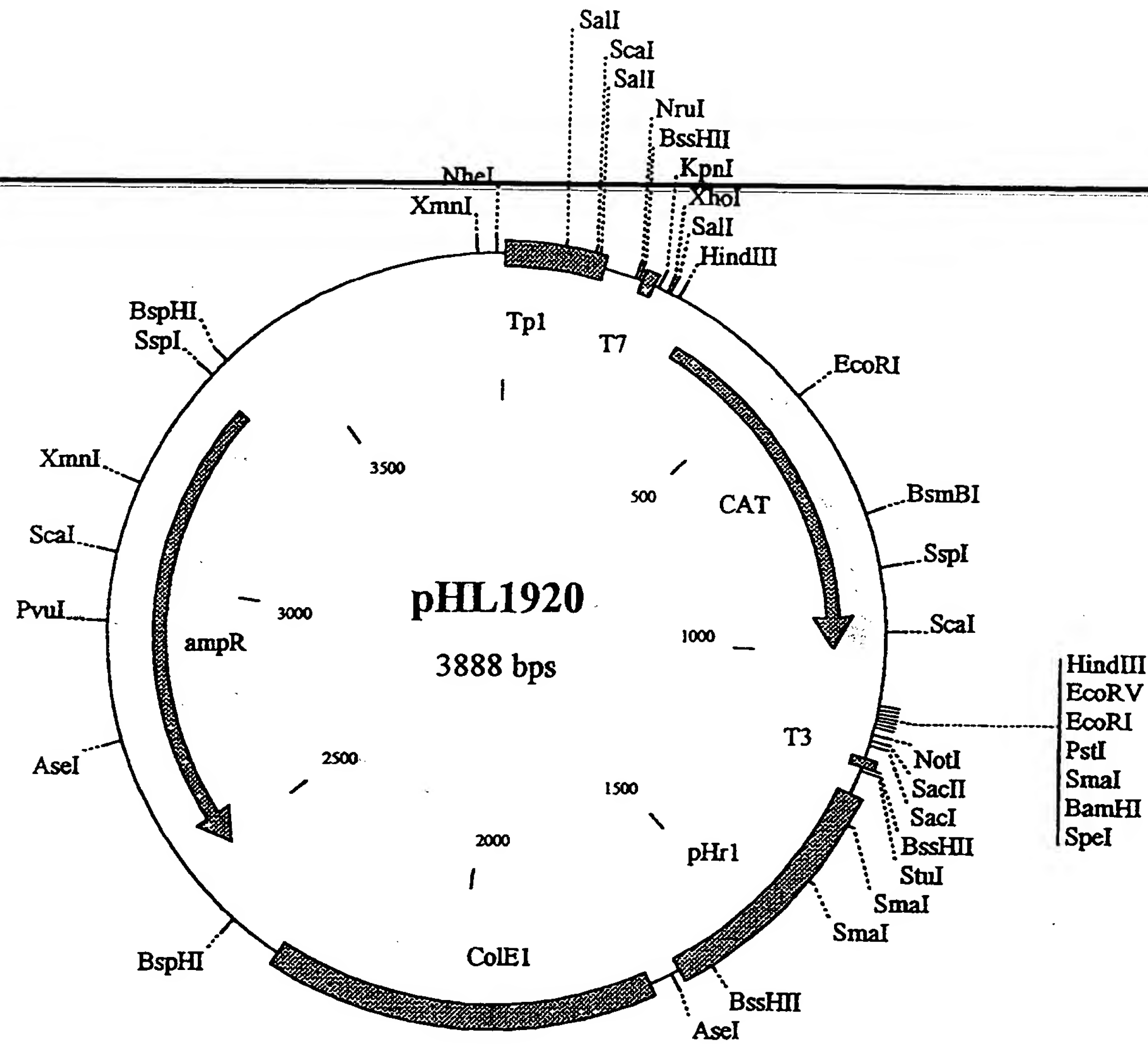

Eco47-3
EaeI
EaeI
EaeI
ApaI
BanII
Bsp120I
PshAI
BspMI
AflIII
DrdI
SspI
DsaI
NcoI
BalI
EaeI
DraI
BsmBI
Esp3I
AlwNI
pHr1
ColE1
3500
3000
500
CAT
BsrDI
ApoI
EcoRI
BspMII
DraI
DraI
pHL 2583
3558 bps
BanI
2500
1000
DraI
PvuII
BsrDI
'ampR
'Tp1
BalI
EaeI
2000
1500
FspI
BsrDI
ampR'
EaeI
NheI
PvuI
EaeI
ApoI
DraI
EarI
SspI

FIG. 19



Eco47-3
ApaI
BanII
Bsp120I
PshAI
SpeI
BspMI
pHr1"
'pHr1'
ColE1
4000
NdeI
BsrGI
BsaAI
PmaCI
BanI
Bst1107
Super-GFP(his)
BsgI
MunI
pHL2989
4343 bps
1000
AsuII
'ampR
FspI
3000
BamHI
XbaI
EcoRV
PvuI
ampR'
"CAT"
2000
PflMI
'Tp1
EcoRI
BspMII
EarI
NheI

FIG. 20


SalI
ScaI
SalI
NruI
BssHII
KpnI
XhoI
SalI
HindIII
NheI
XmnI
Tp1
T7
EcoRI
BsmBI
SspI
ScaI
CAT
500
1000
HindIII
EcoRV
EcoRI
PstI
SmaI
BamHI
SpeI
T3
NotI
SacII
SacI
BssHII
StuI
SmaI
SmaI
1500
pHr1
BssHII
AseI
ColE1
2000
2500
BspHI
AseI
ampR
3000
PvuI
ScaI
XmnI
3500
BspHI
SspI
pHL1920
3888 bps

Abstract

Stable Recombinant Influenza Viruses Free of Helper Viruses

The invention relates to a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus; wherein

(a) at least one of the regular viral RNA segments has been exchanged for a vRNA encoding a foreign gene; and/or

(b) at least one of the regular viral RNA segments is an ambisense RNA molecule, containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement.

The present invention further provides a method for the production of said recombinant influenza virus, a method for constructing specific ribozyme-sensitive influenza carrier strains; pharmaceutical compositions comprising said recombinant influenza virus; and the use of said recombinant influenza virus for preparing medicaments for vaccination purposes.